Nematode inhibitory glutamate-gated chloride ion channel receptors

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NEMATODE INHIBITORY GLUTAMATE-GATED CHLORIDE ION CHANNEL RECEPTORS

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Dedicated to my parents

स्थूलः सूक्ष्मो लघुर्गुरुः व्यक्तो व्यक्तेतरश्चासी

Stūlaḥ sūkṣmoṃ laghur guruh, vyakto vyaktetarascāsi

"Thou art present in different phases, gross and subtle,
light and heavy, manifest and otherwise"-
Kalidasa, Kumarasambhavam, II Canto, Sloka 11.

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ABSTRACT

Alternatively spliced cDNAs derived from the *gbr-2* gene have been amplified by the polymerase chain reaction from the parasitic nematode *Haemonchus contortus*. The *Hc-gbr-2a* mRNA is 1997 nucleotides long; the *Hc-gbr-2b* mRNA is 1317 nucleotides long (accession nos. HCY14233 and HCY14234). The two glutamate-gated chloride ion channel receptor subunits encoded, GBR-2A and 2B have common NH_2-terminal domains but different channel forming membrane-spanning COOH-terminal domains. Sequence analysis suggests that *Hc-gbr-2* is an orthologue of the alternatively spliced *gbr-2* gene cloned from *Caenorhabditis elegans* (Laughton *et. al.*, 1997). The Hc-GBR-2A and 2B subunits exhibit 80% and 84% amino acid identities with Ce-GBR-2A and 2B respectively. The sequence of the *gbr-2* gene from an ivermectin resistant *H. contortus* isolate did not exhibit any differences at the amino acid level. Semi-quantitative PCR analysis of the *Hc-gbr-2a* and *2b* mRNAs shows that *2a* is expressed at much lower levels than *2b* in *H. contortus* embryos. This is in contrast to the *Ce-gbr-2a* and *2b* mRNAs which exhibit similar levels of expression.

An orthologue of the *gbr-2* gene has also been amplified from the parasitic nematode, *Ascaris suum* (accession no. Y18347). However, this does not appear to be alternatively spliced. The As-GBR-2 subunit exhibits highest amino acid identities of 83% and 82% with Hc-GBR-2B and Ce-GBR-2B respectively.

GBR-2 subunits have also been localised in *H. contortus* and *A. suum*. Polyclonal antibodies raised to a peptide matching the sequence of Ce-GBR-2B in the NH_2-terminal domain were used for immunolocalisation. Major part of the staining in both ivermectin susceptible and resistant isolates of *H. contortus* was observed in motor neuron commissures and nerve cords, with some staining on the nerve ring. In *A. suum*, the As-GBR-2 subunit was found on nerve cords. No staining was observed in muscle cells. The results suggest that although the receptor subunits may be orthologous, differences may occur in their expression between nematode species.

CONTENTS

4. Construction and screening of a cDNA library from *Ascaris suum*

5. Amplification of receptor subunit As-GBR-2 from *Ascaris suum*

6. Immunocytochemical localisation of GBR-2 subunits in *Haemonchus contortus* and *Ascaris suum*

Figures

Tables

Abbreviations

Ab	**Ab**ody
Ag	**Ag**en
ACh	**Ac**etyl**ch**oline
APF	**A**rtificial**p**erienteric **f**luid
APS	**A**mmonium **P**er **S**ulphate
ATP	**A**denosine **T**ri **p**hosphate
AVL	**A**nterior **V**entral **L**eft
avr	**Av**ermectin **r**esistance
bp	**B**ase **p**airs
BPB	**B**romo**p**henol **B**lue
BSA	**B**ovine **S**erum **A**lbumin
BZ	**B**en**z**imidazole
CTAB	**C**etyl **T**rimethyl **A**mmonium **B**romide
CTP	**C**ytidine **T**ri-**P**hosphate
dd	**D**eionised **d**istilled
DE	**D**orsal **E**xcitor
DEPC	**D**iethyl **P**yro**c**arbonate
DI	**D**orsal **I**nhibitor
DIC	**D**ifferntial **I**nterference **C**ontrast
DMF	N, N′ **Dim**ethylformamide
DMSO	**Dim**ethyl**s**ul**f**oxide
DNA	**D**eoxyribose **n**ucleic **a**cid
dNTP	**D**eoxy ribo**n**ucleoside **t**ri **p**hosphate
DTT	**D**i**t**hio**t**hreitol
DYT	**D**ouble **Y**east **T**ryptone
eat	**Eat**ing behaviour
EC	**E**ffective **C**oncentration
EDTA	**E**thylene **D**iamine **T**etra **A**cetate
EEO	**E**lectro**e**n**d**o**s**mosis
egl	**Egg l**aying

EGTA	**E**thylene **G**lutaryl **T**etra **A**cetate
ELISA	**E**nzyme-**L**inked **I**mmunosorbent **A**ssay
FITC	**F**luorescein **I**so **T**hio **C**yanate
GABA	γ-**A**mino**b**utyric **a**cid
gbr	**G**ABA receptor
GFP	**G**reen **F**luorescent **P**rotein
GTP	**G**uanidine **T**ri-**P**hosphate
HG	**H**aemonchus **G**ABA
HRP	**H**orse **R**adish **P**eroxidase
IPTG	**I**so-**P**ropyl-**T**hiol-**G**alactoside
IgG	**I**mmunoglobulin **G**
IVR	**I**ver**m**ectin
Kbp	**K**ilo **b**ase **p**airs
Kd	**K**ilo **d**alton
LB	**L**uria **B**roth
lev	**Lev**amisole resistant
LSM	**L**aser **S**canning **M**icroscope
MAB	**M**onoclonal **A**nti**b**ody
M-MLV	**M**oloney **M**urine **L**uekemia **V**irus
MOPS	(N-**m**orpholin**o**) **p**ropanesulfonic acid
mRNA	**M**essenger RNA
nAChR	**N**icotinic **A**cetyl**ch**oline **R**eceptor
NZY	**NZ**-amine-**Y**east
O.D.	**O**ptical **D**ensity
PAGE	**P**oly**a**crylamide **G**el **E**lectrophoresis
PBS	**P**hosphate **b**uffered **s**aline
PCR	**P**olymerase **C**hain **R**eaction
PFA	**P**ara**f**orm**a**ldehyde
PIPES	**Pi**perazine-N, N′-bis [2-ethanesulfonic acid]
Pfu	**P**laque **f**orming **u**nits
pm	**P**haryngeal **m**uscle
RACE	**R**apid **A**mplification of **c**DNA **E**nds
RIS	**R**ing **I**nterneuron **S**ynapse

RMED	**R**ing **M**otoneuron **E**xcitatory **D**orsal
RMEV	**R**ing **M**otoneuron **E**xcitatory **V**entral
RMG	**R**ing **M**otoneuron **G**anglion
RNA	**R**ibo**n**ucleic **a**cid
RNase	**R**ibo**n**uclea**se**
RNasin	**R**ibo**n**uclea**se** **in**hibitor
rNTP	**R**ibo**n**ucleoside **T**ri **P**hosphate
RT	**R**everse **T**ranscriptase
SDS	**S**odium **D**odecyl **S**ulphate
SL	**S**pliced **L**eader
SM	**S**odium chloride, **M**agnesium chloride
ss	**S**ingle **s**tranded
SSC	**S**tandard **S**aline **C**itrate
TBE	**T**ris-**B**orate-**E**DTA
TE	**T**ris-**E**DTA
TEMED	N, N, N′, N′,-tetra**me**thylene **d**iamine
TM	**T**rans-**M**embrane
Tm	**M**elting **T**emperature
TMB	3, 3′, 5, 5′ Tetra**m**ethyl **B**enzidine
Tris	**Tris**-(hydroxymethyl)-aminomethane
TRIT-C	**T**etramethyl**r**hodamine **I**sothio**c**yanate
TTP	**T**hymidine **T**ri **P**hosphate
unc	**Unc**oordinated
UTR	**U**ntranslated **R**egion
VI	**V**entral **I**nhibitor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-**gal**actosidase
YAC	**Y**east **A**rtificial **C**hromosome

1. INTRODUCTION

1.1 Helminth Infections

The term helminth derived from the Greek words *helmins* or *helminthos*, literally means 'worm'. This includes invertebrates with an elongate body without appendages, and often without a clearly differentiated head-region. Infections caused by these worms are a major cause of economic loss and public health problems. Intestinal parasitic nematodes alone account for a third of helminth infections in humans world-wide. Approximately 1.3 billion people for instance are estimated to be infected with the roundworm, *Ascaris lumbricoides* (de Silva *et. al.*, 1997). Other nematode infections include trichuriasis caused by *Trichuris trichiura* (whipworm); hookworm infections caused by *Ancylostoma duodenale* and *Necatar americanus*; onchocerciasis or river blindness caused by *Onchocerca volvulus*; lymphatic filariasis caused by *Wucheria bancroftii* and *Brugia malayi* and dracanculensis caused by *Dracanculus medenensis* (guinea worm), all of which affect nearly 2 billion people (WHO, 1997).

Parasitic nematodes are also known to occur in animals and plants. *Dirofilaria immitis* infecting dogs; *Nippostrongylus brasiliensis* in rats; *Ascaris suum* in pigs; and *Haemonchus contortus* and *Ostertagia circumcincta* infecting sheep and goats are few examples. Among parasitic nematodes of veterinary importance *H. contortus* infections result in heavy losses in livestock production. The Australian livestock industry for instance incurs a loss in production of nearly AUS $200 million due to this helminth infection (Mcleod, 1995). Severe losses in crops are known to occur by plant parasitic nematode infections. An important example is that of the potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*, pathogenic to the commercial potato *Solanum tuberosum* (Duncan *et. al.*, 1997).

1.2 Parasitic nematodes

Despite the diversity and complexity of their life cycles, the animal parasitic nematodes share certain common features in general. Externally the nematodes are cylindrical and are covered with a cuticle (Maizels *et. al.*, 1993). This is secreted by the hypodermis and consists of a syncytium of cells with four longitudinal thickenings- the lateral cords

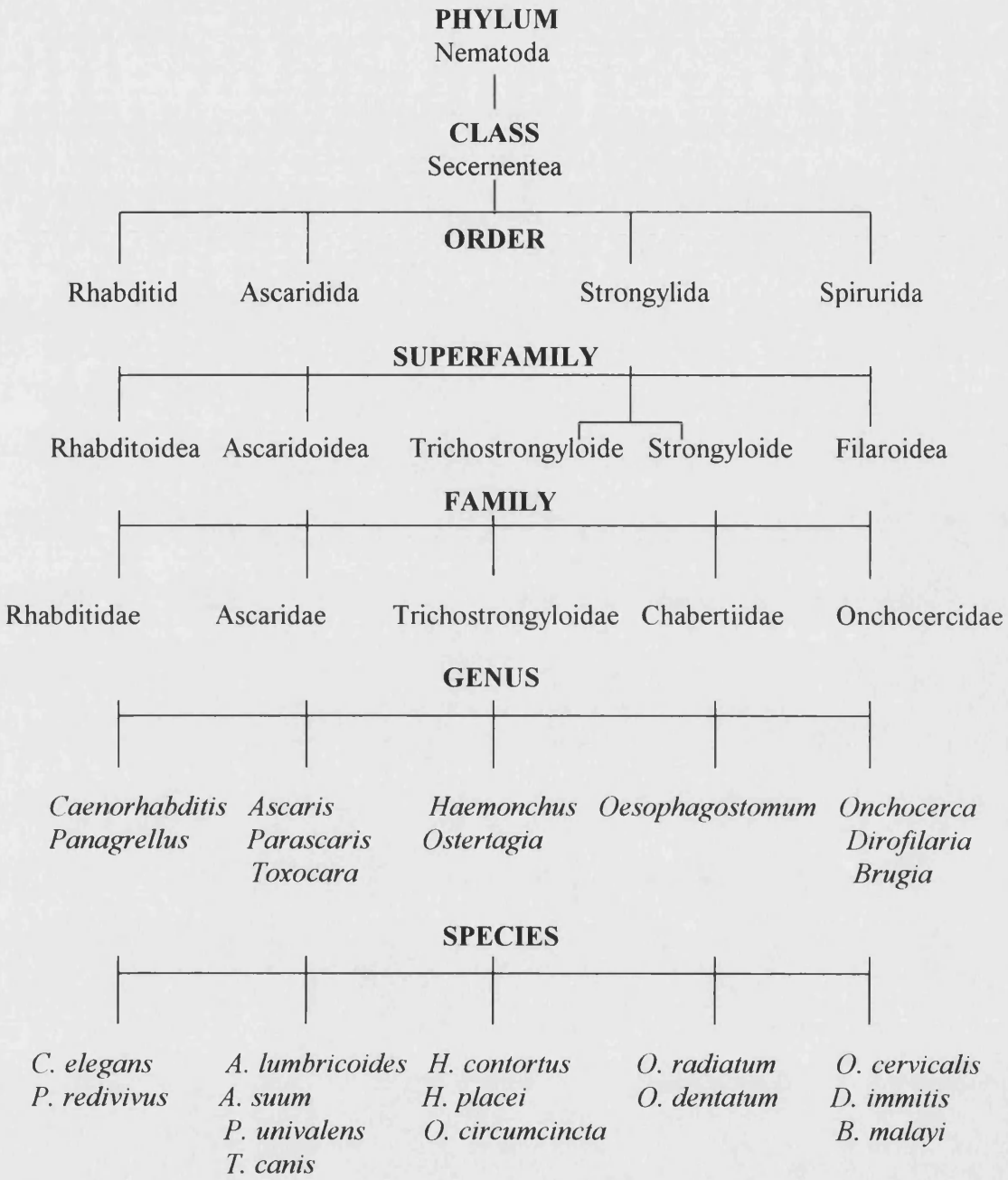


Figure 1.1 Classification of nematodes mentioned in the present study.

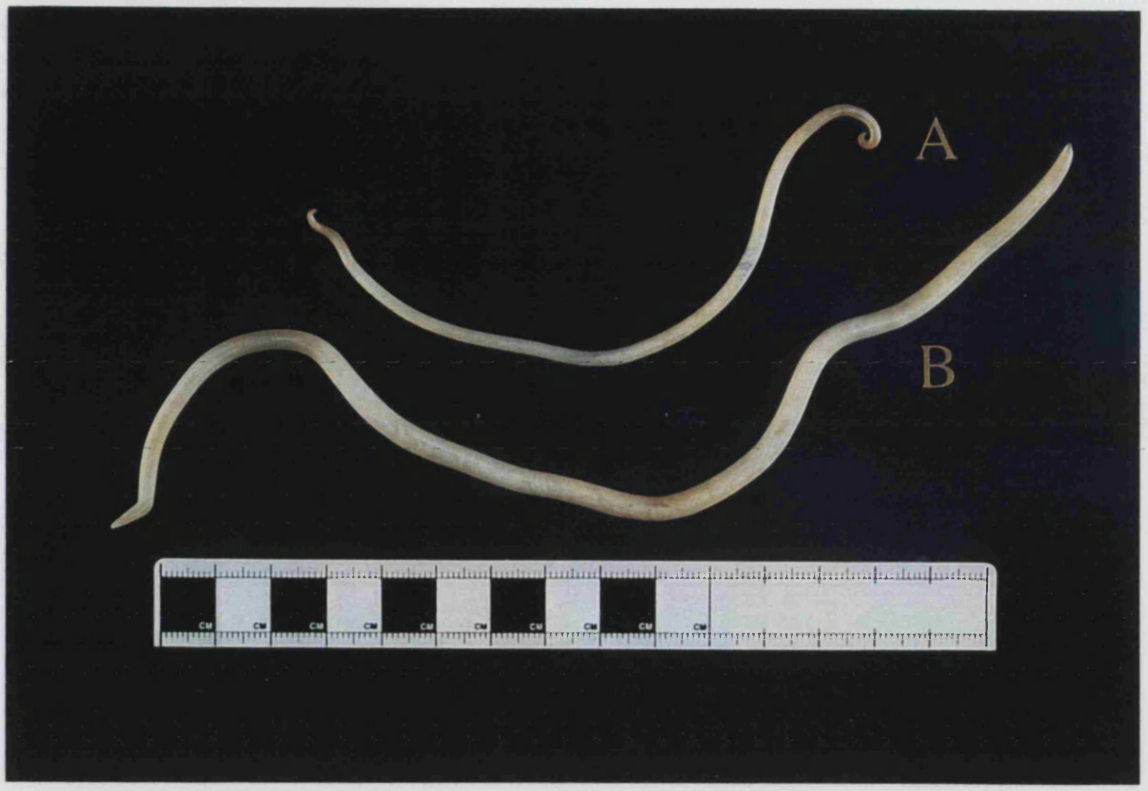
containing the excretory canals and some lateral nerve trunks; and the dorsal and ventral cords carrying the major nerve trunks (Wharton, 1986). The cords separate the layer of longitudinal muscles inside the hypodermis into four major groups. A coelomic fluid fills the body cavity. The nematode internal structures include a digestive tract consisting of the mouth, leading into a muscular pharynx which pumps food into the intestine, followed by a rectum. The excretory system consists of two unbranched lateral canals. These are joined at the anterior end by an excretory duct which leads into an excretory pore. The nervous system consists of a ring of ganglia surrounding the pharynx. Nerve trunks pass from this ring into the dorsal and ventral nerve cords. A second set of ganglia is present at the posterior end.

The reproductive life cycles of most nematodes involve separate sexes. Sexual reproduction occurs within an infected host. Eggs are laid by the female which are then passed outside the host. The first stage L1 larvae develop within the egg and pass through two growth and moult cycles. The resulting infective L3 larvae are ingested by the host, where they develop into L4 larvae and adult males or females.

1.2.1 *Ascaris suum*

Ascaris suum, which infects pigs, is closely related to the human parasite, *Ascaris lumbricoides*. The taxonomic status of the two species has been debatable. The two nematodes have been referred to either as separate species or subspecies. However, Nadler (1987) observed from biochemical studies that *Ascaris suum* and *A. lumbricoides* were sibling species. This nomenclature is followed in the present study. An outline of the classification is shown in Figure 1.1.

Adult *A.suum* females are 25-40cm long compared to males of size 15-30cm (Figure 1.2). The males bear a curved posterior end and a slit-like anal opening. A schematic outline of its lifecycle in pigs is shown in Figure 1.3. Eggs laid by adult female worms in the pig gut are passed out with the faeces. Under suitable soil conditions infective larvae develop inside the eggs. These hatch only in the definitive host when ingested and result in infection. The larvae finally moult into mature adult worms (Smyth, 1994).



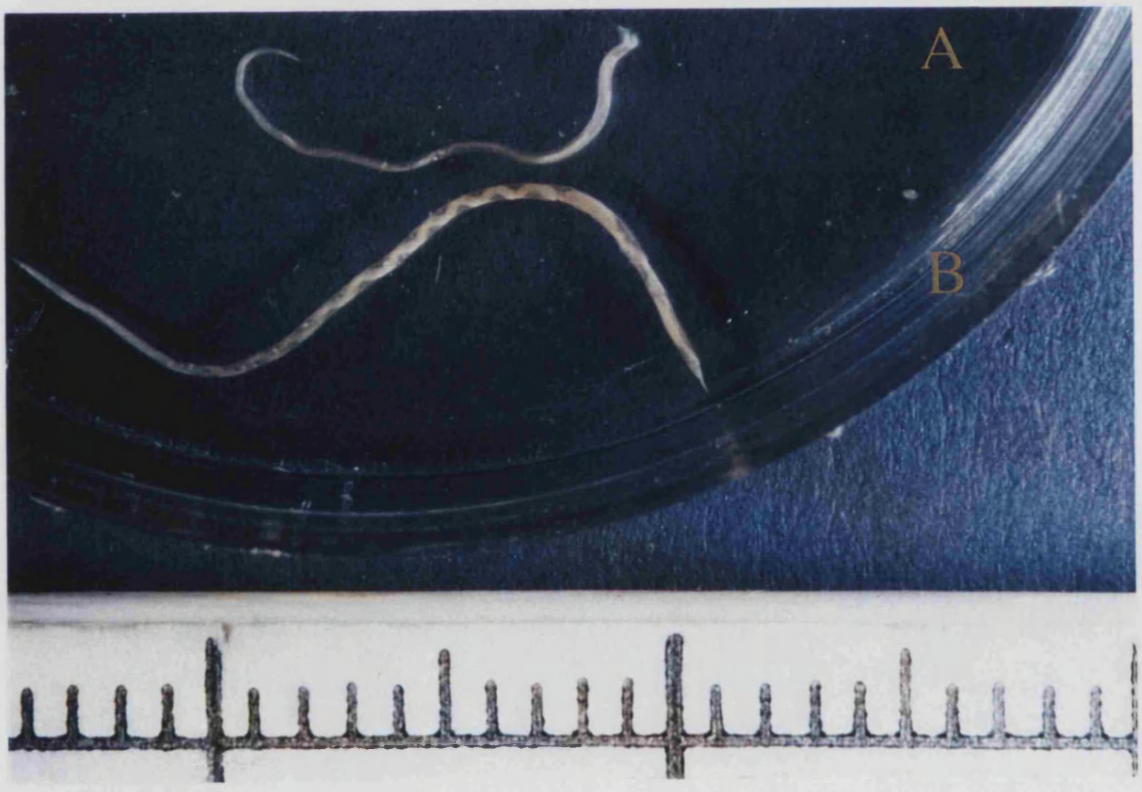


Figure 1.2 (above) *Ascaris suum* **(A)** Male **(B)** Female.
Figure 1.3 (below) *Haemonchus contortus* **(A)** Male **(B)** Female.

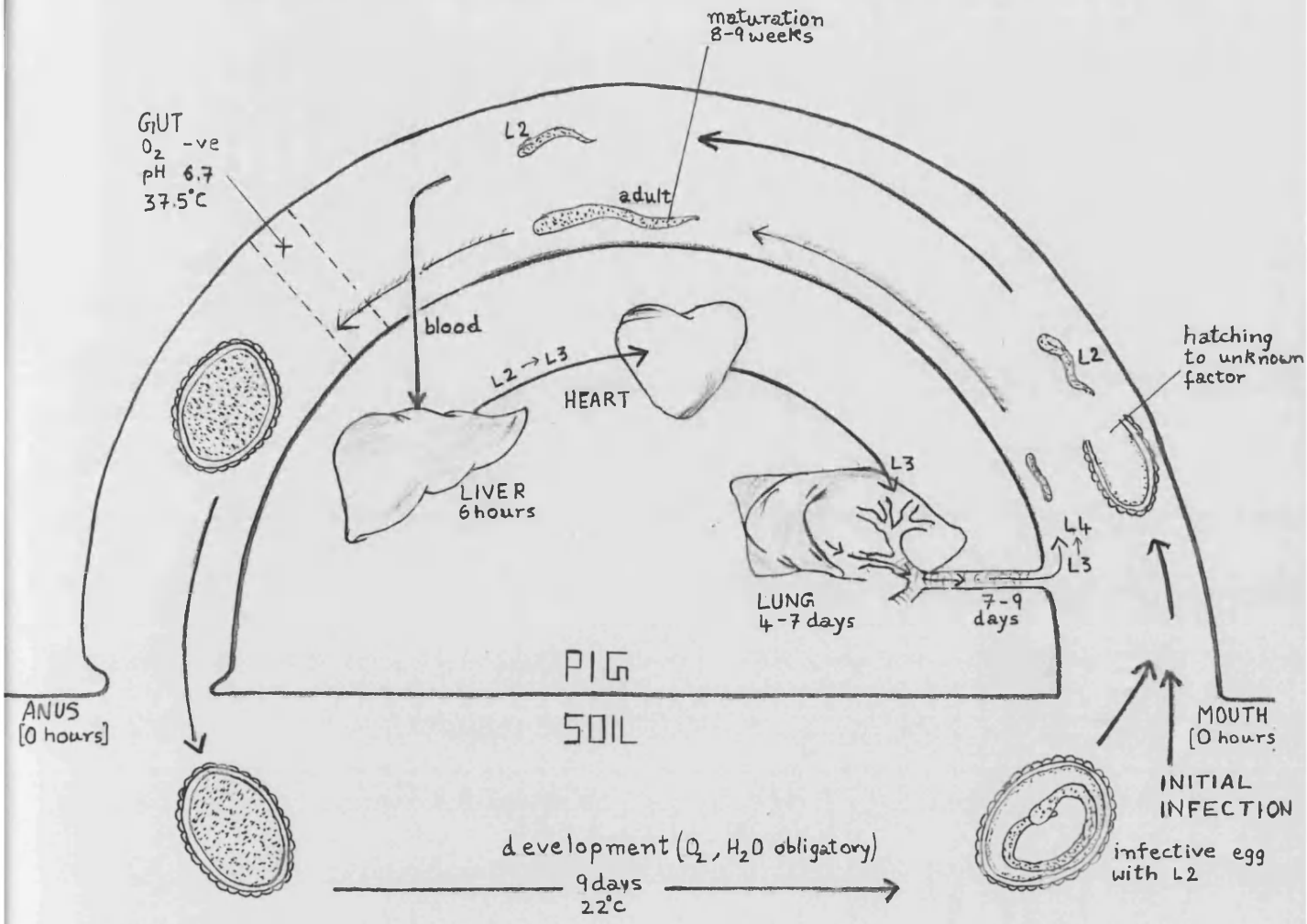


Figure 1.4 Schematic of the lifecycle of *Ascaris suum*.

A. suum infection results in pneumonitis, bronchial irritation, liver pathology, and other associated symptoms related to porcine ascariasis. Migration of the larvae through the lungs also results in haemorrhage of the blood vessels. Due to the genetic similarities between *A. lumbricoides* and *A. suum*, there is building evidence of pig *Ascaris* as an important human zoonose (Murrell *et. al.*, 1997; Maruyama, 1996). The repeated exposure to *A. suum* eggs from pig manure is suggested to result in frequent larval invasion of the human liver (Anderson *et. al.*, 1995). Severe human infections caused by *A. suum* have been implied as a result of similar larval migratory behaviour in pigs and humans (Crompton, 1994).

1.2.2 *Haemonchus contortus*

H. contortus is found in the abomasum of ruminants and feeds on blood. Adult females are 18-30mm in size and larger than the males of size 10-20mm (Figure 1.3). The female has a striking appearance of a barber's pole as its white ovaries are wrapped around a red blood filled intestine. Males are characterised by the presence of a hand like structure called the copulatory bursa and brown spicules (Figure 1.6). Adult worms lay eggs in the sheep gut which pass out with the faeces. About 10,000-15,000 eggs are laid per day (See Appendix V). Ensheathed L3 larvae unable to penetrate the skin must be ingested from soil or blades of grass to cause infection. A schematic of its life cycle is shown in Figure 1.5. Infection results in severe weight loss of the animal and anaemia.

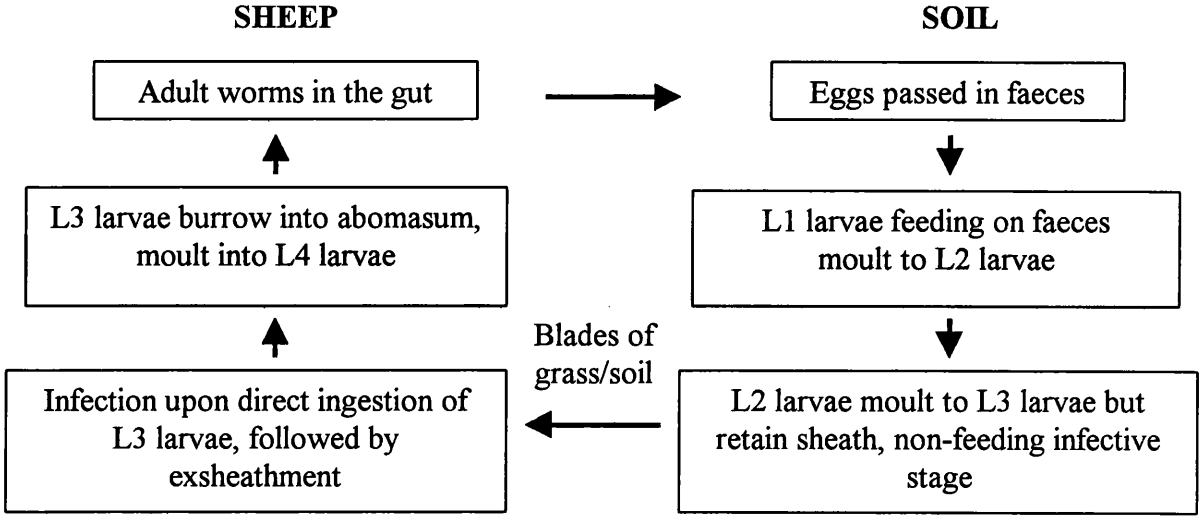


Figure 1.5 Schematic of life cycle of *Haemonchus contortus*.

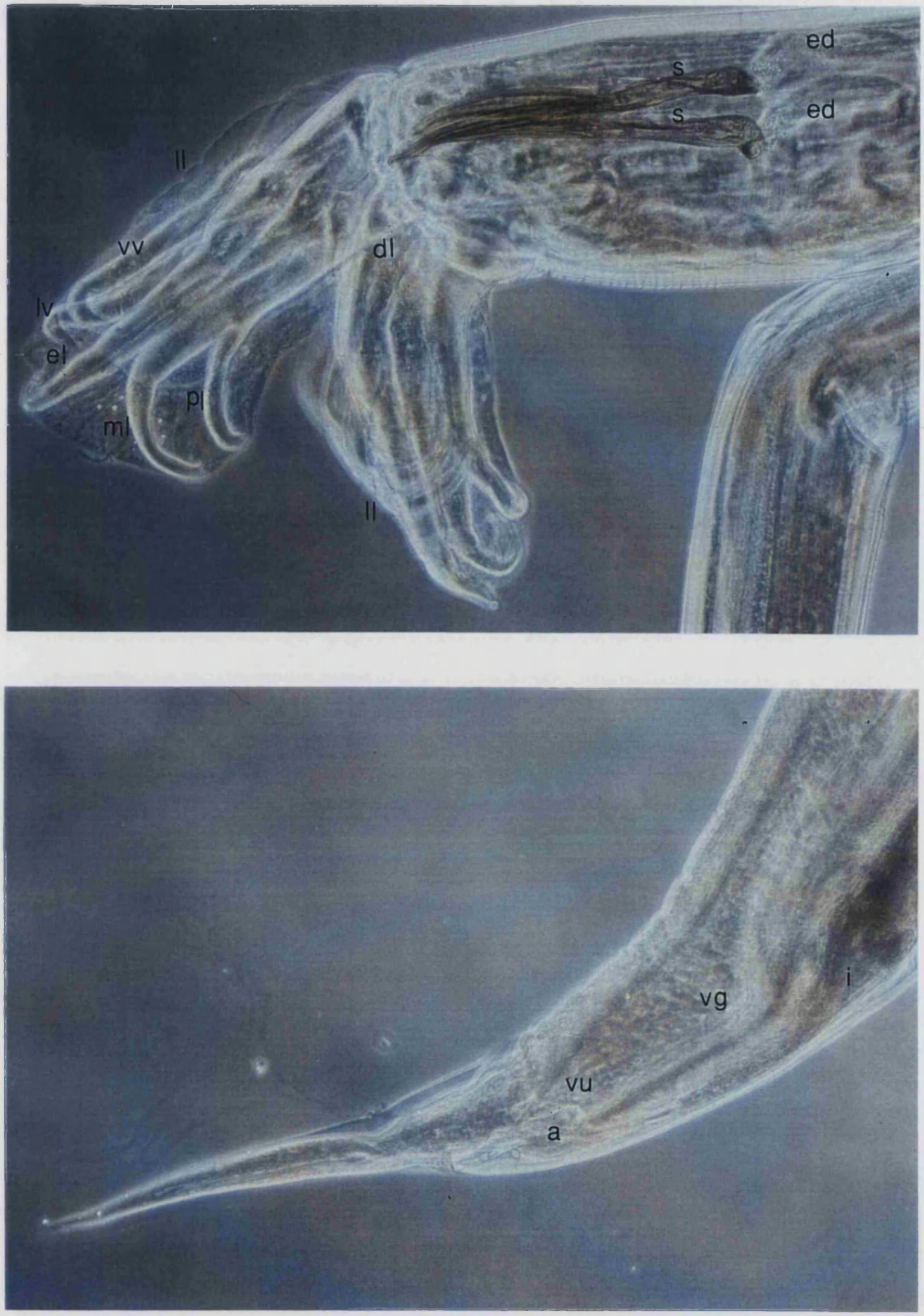


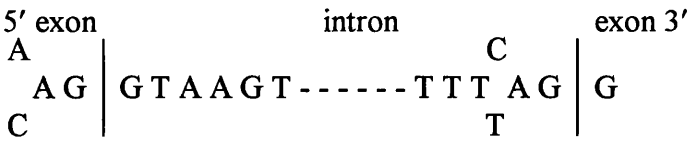
Figure 1.6 Caudal regions of *Haemonchus contortus* **(A)** (above) male and **(B)** (below) female. The copulatory bursa of the male is distinct as it has two large lateral lobes (ll) and a small dorsal lobe (dl). The left lobe rays are more divergent than the right. The rays are- ventro-ventral (vv), latero ventral (lv), externo-lateral (el), medio-lateral (ml) and postero-lateral (pl). The distal end of the ejaculatory duct (ed) containing two brown spicules (s) can be seen. In the female, the uterus is connected to a muscular ovijector leading into the vagina (vg) and the vulva (vu). The intestine (i) leading to the anus (a) can be seen running alongside.

1.3 A Free living nematode, *Caenorhabditis elegans*

Most nematode species are free-living. *C. elegans* is a soil nematode about 1mm long. It is an attractive model experimental system (Hodgkin *et. al.*, 1995). It has a simple life cycle with a rapid three day generation time, and is completely transparent during all the developmental stages (Brenner, 1974; Wood, 1988). Its genome is small (8×10^7 base pairs) and a virtually complete physical map in the form of cosmids and YAC (yeast artificial chromosomes) clones exist. The adult hermaphrodite contains a specific number of 959 somatic nuclei. Its nervous system is particularly well defined with 302 neurons and a well mapped cell lineage (White *et. al.*, 1986). Such features of *C. elegans* have made it a popular model system for parasitic nematodes (Bürglin *et. al.*, 1998). The 'wiring diagram' of its nervous system is for example, largely similar to that of *A. suum* (Stretton *et. al.*, 1978; White *et. al.*, 1976). In addition, the *C. elegans* genome project has particularly facilitated rapid identification of conserved orthologous parasite genes (Kwa *et. al.*, 1995). *C. elegans* can also be used as a heterologous system to study functional aspects of parasite genes. However, there may be differences as the phenotypic constraints of a parasitic life style do not apply to the free living nematode. In addition, electrophysiological and pharmacological studies possible on large nerve and muscle cells of *A. suum* have not been feasible in smaller nematodes, making direct comparisons difficult (Stretton *et al.*, 1978; 1992) although recently *in situ* patch clamp recordings from *C. elegans* neurons have been made by Goodman *et. al.*, (1998).

1.4 Parasitic nematode genomes

The genomes of parasitic nematodes are unusually small compared to other animal genomes. Typically, the size of the haploid genome is in the order of 10^8 bp. Most nematode genomes are A-T rich, the filariid parasites having some of the most A-T rich genomes (Hammond and Bianco, 1992). The coding regions of the genomes (exons) are often short. The intervening non-coding regions (introns) can be as short as 50bp. Consensus sequences at intron-exon junctions from parasitic nematodes, as in *C. elegans* (Wood, 1988), often match the sequence:



Both *cis* and *trans* splicing of the mRNAs are known. Of interest is the presence of a 22 nucleotide spliced leader sequence SL1, added at the 5′ end of most nematode mRNAs by a *trans*-splicing mechanism (Nilsen, 1993). The leader sequence comes from the 5′ end of a 100 nucleotide non-polyadenylated RNA. In *Ascaris*, approximately 80%-90% of mRNAs acquire the spliced leader (Davis, 1996). The *trans* splicing serves as a post-transcriptional mechanism of regulation, although its exact functional role is not very clear (Nilsen, 1995).

Another feature of the nematode genome is the property of chromatin diminution. This is a complex mechanism of chromosome fragmentation. It involves heterochromatin elimination, addition of new telomeres and degradation of DNA in presomatic cells during early embryonic development (Muller *et. al.*, 1996). This developmentally controlled genome rearrangement is observed in some, but not all parasitic nematodes. Of the eleven nematode species in which it is reported, most belong to the Ascarididae family, for example, *Ascaris* (Streeck *et. al.*, 1982), *Parascaris univalens* and *Toxocara canis* (Esteban *et. al.*, 1995). In *A. suum*, about 25% of the total nuclear DNA of approximately 1.6 x 10^5 Kb, is cast out from the somatic cell lineages but retained in the germ line (Tobler *et. al.*, 1972). This has also added to the difficulty in determining the exact chromosome number (Tobler *et. al.*, 1992). There is no evidence of chromatin diminution in *C. elegans* or other free-living nematodes. The reasons as to why some nematodes undergo the process while others do not, is still not clear. However, it is possible that the radical 'gene-throw-away-approach' serves as an alternate means of gene regulation, especially at the transcriptional level (Etter *et. al.*, 1994).

1.5 Nematode functional organisation

1.5.1 The pharynx

Nematodes are mainly liquid feeders. They feed on liquids directly (blood, plant juices) or on particles ingested in a liquid medium (bacteria, tissue fragments). Feeding is

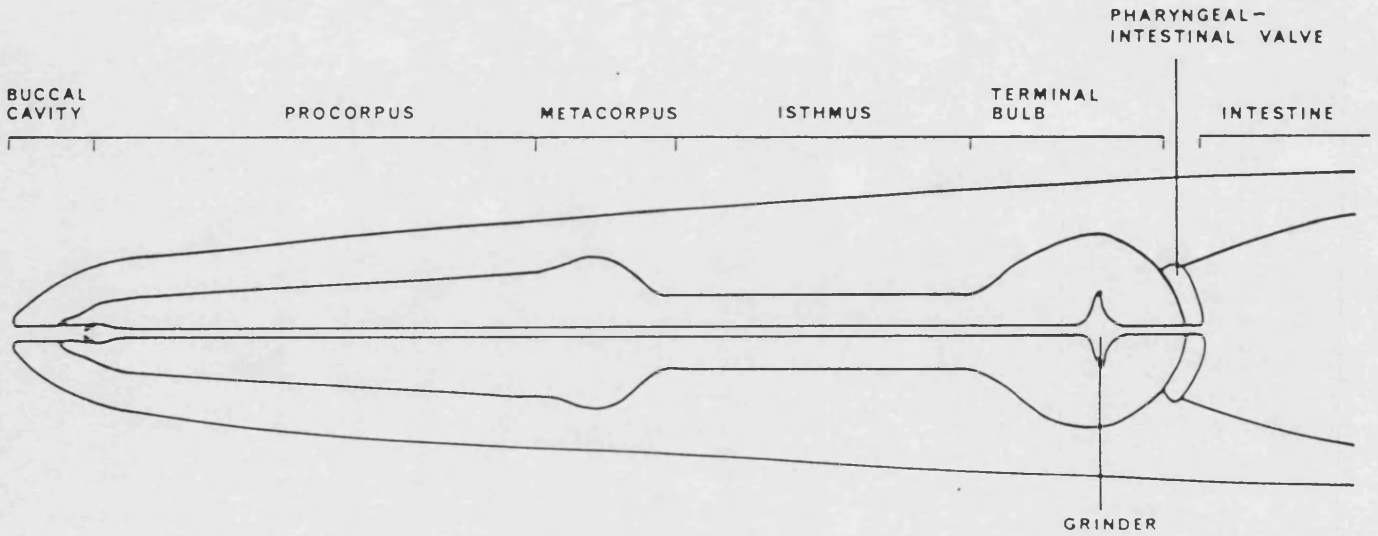


Figure 1.7 Schematic of the pharynx in *C. elegans*.

aided by a pharyngeal pumping mechanism. The structure of the pharynx is variable and consists of a variety of swollen muscular bulbs or isthmuses. It can be considered as two concentric cylinders (the outer wall and the lining of the lumen), connected by radial muscles. The pharynx is also well connected to the nervous system. A basement membrane isolates the pharynx from the rest of the animal, thus making the pharyngeal nervous system a nearly self-contained unit containing motor neurones and interneurones. When the muscles contract, intrapharyngeal pressure builds up, which propels liquid or suspended food particles into the lumen (Wharton, 1986). The pumping behaviour of the pharynx is also partly controlled by the nervous system (Brownlee *et. al.*, 1994a). Upon relaxation of the muscles, the contents are forced into the intestine through the pharyngeal intestinal valve.

The pharynx in *C. elegans* is composed of four distinct regions– the anterior procorpus and a bulb-shaped metacorpus which filter the food particles, mainly bacteria, from the surrounding medium and then transport it to a cylindrical isthmus. This leads into a terminal bulb which grinds up the bacteria and passes the debris into the intestine (Figure 1.7) Different cell types constitute these regions. Of the 60 cells in the pharynx, 20 are muscle cells of 8 anatomical types, 20 are neurons of 14 anatomical types, and the rest are structural and glandular cells. The muscle cells constitute eight different layers of pharyngeal musculature (Albertson and Thomson, 1976). Table 1.1 lists the number of cells and nuclei in each of the muscle cell types.

Muscle cell type	Number of cells	Number of nuclei
m1	1	6
m2	3	3
m3	3	6
m4	3	6
m5	3	6
m6	3	3
m7	3	3
m8	1	1

Table 1.1 Number of cells and nuclei in each pharyngeal muscle cell of *C. elegans*.

In *A. suum*, the pharynx is cylindrical, and lacks a bulbar enlargement. Its pumping behaviour occurs as a result of an action potential causing contraction of the radial muscles (Del Castillo and Morales 1967b). A negative-action potential due to a potassium current results in relaxation, and hence a 'power stroke', transferring the food particles into the intestine (Byerly and Masuda, 1979). The exact mechanism of the control of pharyngeal function is, however, still unknown (Brownlee, 1995).

In *H. contortus*, the tube-like pharynx is simple, with little or no differentiation. Apart from the description of the anatomy by Veglia (1915), little is known about its structure in detail. A cross section of the pharynx shows triradiate symmetry and a cuticle lined lumen with the oesophageal muscle around it (Sood and Sehajpal, 1978; Weise; 1977).

1.5.2 The neuromuscular system

Nematode movement is controlled by the neuromuscular system. Locomotion occurs by generation and propagation of a body waveform which consists of an alternating series of dorsal and ventral bends. The bend at any point is generated by contraction of dorsal or ventral muscles, followed by simultaneous relaxation of the opposing muscles (ventral and dorsal respectively). This results in a sinusoidal motion (Wharton, 1986). The body movements of most nematode parasites allow them to maintain their position in the host animal, but alter the position of the head for feeding. The neuromuscular connections involved have been examined in *A. suum* (Rosenbluth, 1965) and *C. elegans* (White *et. al.*, 1976).

Ascaris bears around 300 neurons whose position and structure are completely reproducible form animal to animal (Goldschmidt, 1908). In the head region, a circumferential nerve ring is located 1-2mm posterior to the anterior tip of the worm. The nerve ring surrounds the pharynx. Two major longitudinal nerve cords, the dorsal and the ventral cords arise from this ring, and extend down to the tail, where a second ganglia network exists. The cords are separated by swellings of the hypodermis called lateral lines. In the immediate vicinity of the nerve ring are several loosely organised ganglia, including the dorsal, ventral, and lateral ganglia. A retrovesicular ganglion is located within the ventral nerve cord approximately 2mm posterior to the nerve ring (Angstadt *et.*

al., 1989). The nerve cords contain cell bodies and processes of motor neurons which make synapses to the muscle (Stretton *et. al.*, 1978). Seven classes of motor neurons have been identified with neuromuscular output either into the dorsal cord or the ventral cord. While three classes of motor neurons DE1, DE2 and DE3 excite the dorsal muscle, the DI and VI motor neurons inhibit the dorsal and ventral muscles respectively (Walrond *et. al.*, 1985). All the five types of motor neurons have processes in both the dorsal and the ventral nerve cords. Two classes of motor neurons V-1 and V-2 are putative ventral exciters. A lateral dorsoventral neuronal process called a commissure extends between the nerve cords and links the processes coming out of each cell type. The commissures are embedded in the hypodermal tissue. The motor nervous system also constitutes interneurons that occur exclusively in the ventral cord. The ventral processes of DE1, DE2, and DE3 motor neurons receive synapses from the interneurons. The synaptic organisation thus allows for propagation of waves of muscle contraction upon stimulation of a single excitatory motor neurone (Stretton *et. al.*, 1992).

The neuromuscular connections of nematodes are unusual in that the muscle sends out a process that synapses with the motor neurons in the nerve cord rather than vice-versa. This has been demonstrated in *Ascaris* (Rosenbluth, 1965) and *C. elegans* (White *et. al.*, 1976). Figure 1.8 shows a transverse section through the anterior region of *A. suum*. Each muscle cell is composed of three parts, an elongated fibrillar contractile region known as the 'spindle'; a balloon shaped bag structure of 200 μm diameter, bearing the nucleus of the cell with submembrane mitochondria and particulate glycogen; the arm, a thin process reaching transversely from the bag to one of the longitudinally running nerve cords (Del Castillo *et. al.*, 1964a) (See also Appendix V). Each somatic muscle cell has an average of 2.7 arms. The arms reach the syncytium, where they break into a number of fine processes called 'fingers'. The arms come together to form a syncytium over the nerve cord and are electrically coupled (De Bell *et al.*, 1963).

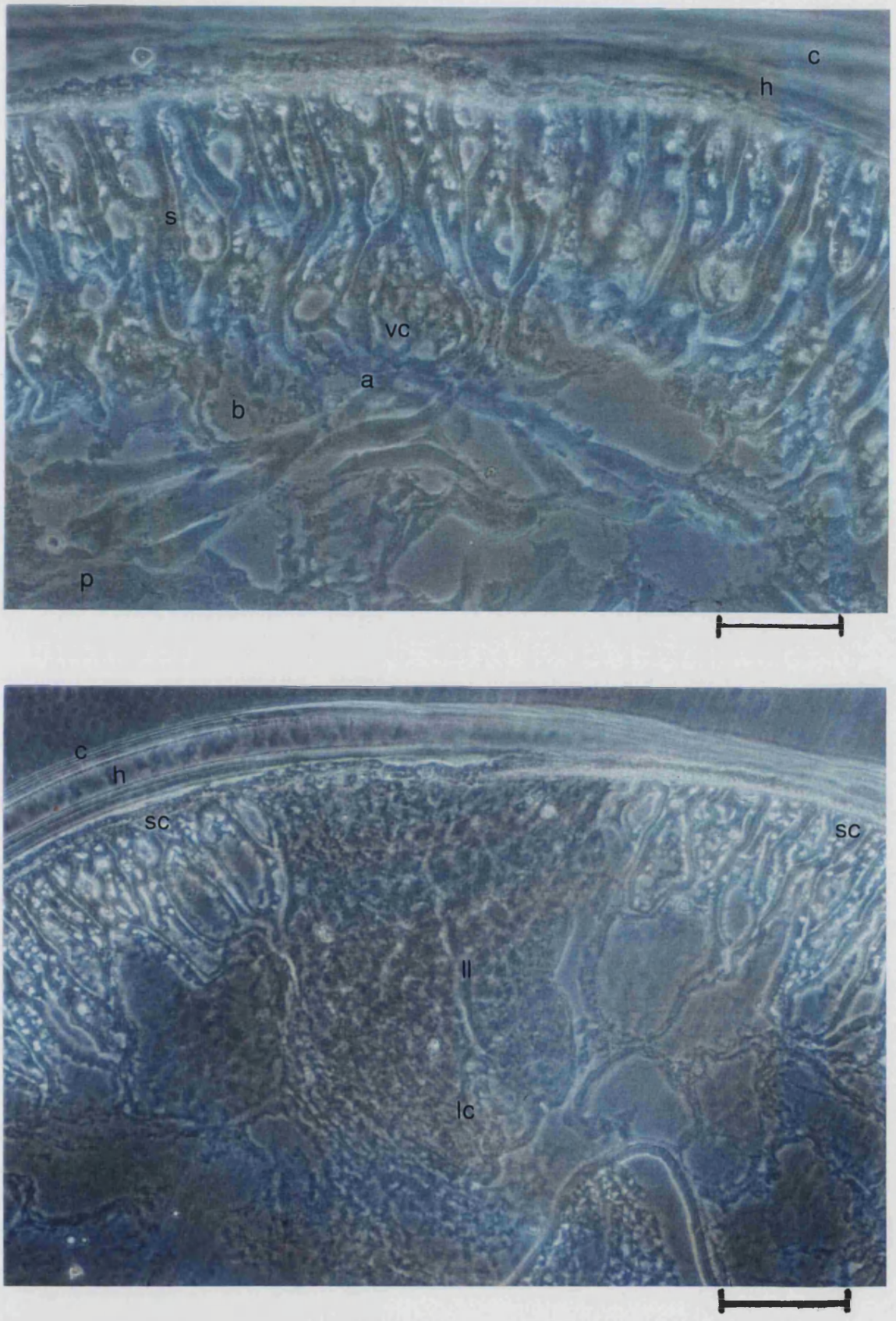


Figure 1.8 Phase-contrast micrographs of a transverse section through the anterior region of *Ascaris suum*. **(A)** (above) Detail in the region of the ventral cord (vc). muscle spindle (s); belly (b); muscle arm (a); hypodermis (h); outer and inner lamella of cuticle (c); perienteric fluid (p). **(B)** (below) Detail in the region of the lateral line (ll). lateral canal (lc); sub-dorsal cords (sc). Scale bar = 50μm.

1.6 Control of helminth parasites

1.6.1 Antiparasitic drugs

Parasitic helminths cause severe infections resulting in morbidity and mortality in humans and farm animals. Several control measures have been investigated. The use of antiparasitic chemicals appears to be the most effective and is the most widely used. The design of anthelminthics has involved no 'rational' design (Gutteridge, 1997). Drugs isolated from both natural botanic and microbiological resources or synthetic processes have been screened at random thus far (Thompson *et. al.*, 1996). Most drugs act by interfering with the cellular physiology or by disrupting the nematode energy- metabolism, or by selectively acting on the neuromuscular system of the parasite (Geary *et. al.*, 1992). On the basis of their chemistry, the broad-spectrum drugs effective against several parasites can be divided into three major groups. Their modes of action differ. These are- 1) the benzimidazoles, 2) imidothiazoles and tetrahydropyrimidines, and 3) avermectins. The benzimidazole drugs such as thiabendazole, mebendazole and albendazole were the earliest in use (Figure 1.9A). They bind free β-tubulin selectively in the parasite and prevent polymerization of tubulin and microtubule-dependent uptake of glucose, (Lacey and Gill, 1994). How the drugs act exclusively on the nematode and not on the host is still not clear.

The second group of drugs includes imidothiazoles like levamisole, and tetrahydropyrimidines such as tetramisole, pyrantel and morantel (Figure 1.9B). These drugs act on the nicotinic receptors of nematodes causing depolarisation and an increased input conductance of the muscle membrane to sodium and potassium. This results in spastic paralysis of the parasite, causing its expulsion (Martin *et. al.*, 1997).

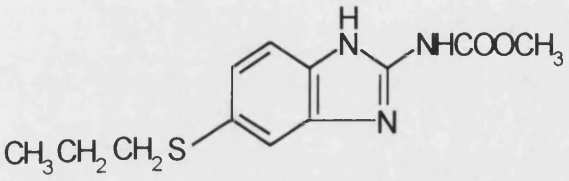
The more recently introduced avermectin class of anthelminthics are macrocyclic lactones, derived from the soil mould *Streptomyces avermectilis* (Campbell, 1991). These include the drugs ivermectin, abamectin, doramectin, moxidectin and milbemycin D (Conder and Campbell, 1995) (Figure 1.9C). Ivermectin, (22, 23 dihydroavermectin B_{1a}), a semi-synthetic derivative of avermectin, is of particular interest due to its broad spectrum of activity against nematodes and arthropods, and low toxicity against the vertebrate host (Shoop, 1993). It was suggested to interact with receptors at chloride channels (Holden-Dye and Walker, 1990). However, the exact identity of the target ion channel has been controversial (Arena, 1994). The mode of action initially suggested to

be via GABA gated chloride ion channels, is now attributed to glutamate gated chloride ion channels (Arena *et. al.*, 1995).

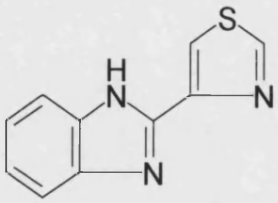
Figure 1.9 Chemical structures of three major groups of anthelminthics: **(A)** Benzimidazoles **(B)** Imidothiazoles and **(C)** Avermectins. (Drawn using ISIS DRAW, version 2.1.3).

(A) Benzimidazoles

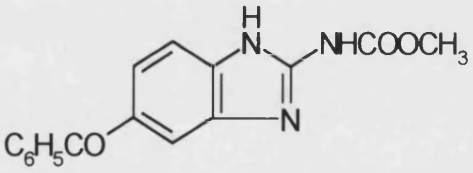
Albendazole



Thiabendazole

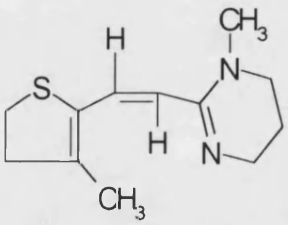


Mebendazole

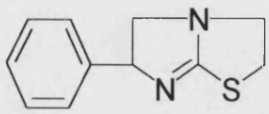


(B) Imidathiazoles

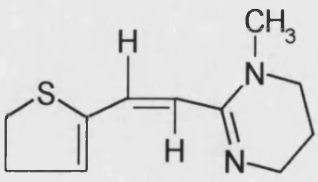
Morantel



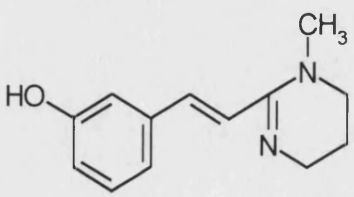
Levamisole



Pyrantel

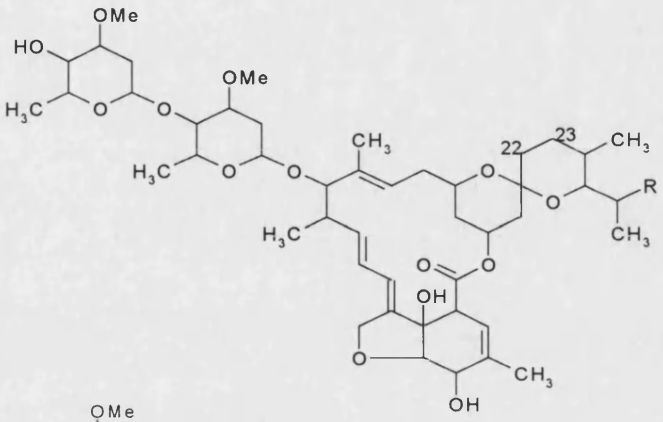


Oxantel

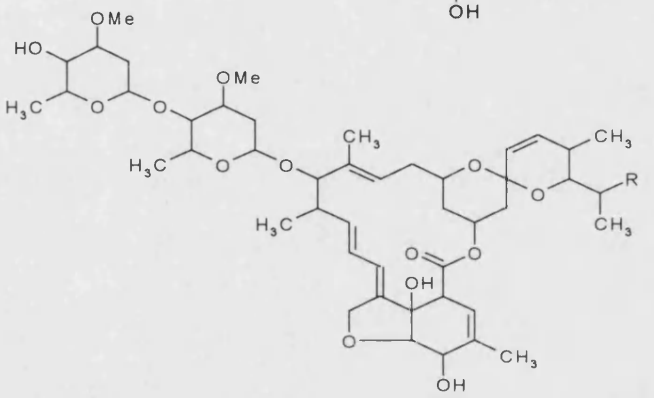


(C) Avermectins

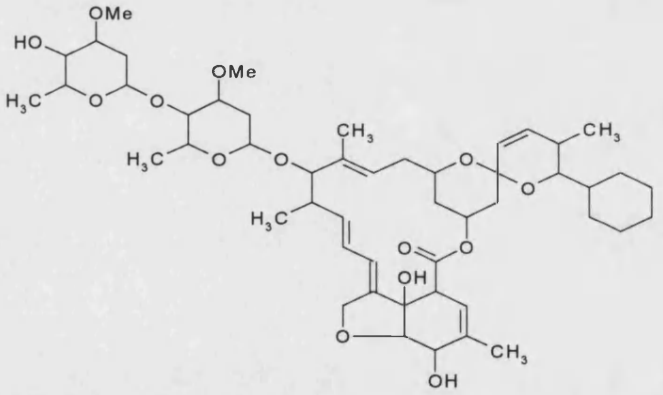
Ivermectin: 80% 22, 23 dihydroavermectin B_{1a} (R is sec-butyl) and 20% 22, 23 dihydroavermectin B_{1b} (R is iso-propyl).



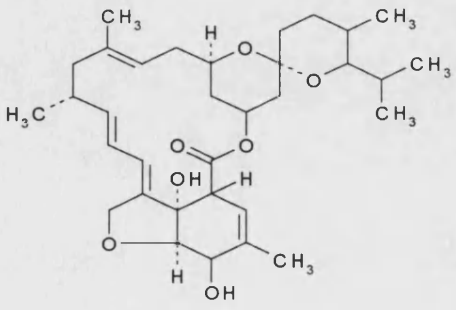
Abamectin



Doramectin



Milbemycin D



1.6.2 Development of drug resistance

Abundant use of anthelminthics has resulted in the selection of drug resistant parasitic nematode populations in livestock animals, particularly in trichostrongylid nematodes (Hong *et. al.*, 1996; Le Jambre *et. al.*, 1995; Van Wyk *et. al.*, 1997). Resistance is now reported in all three groups of broad-spectrum drugs. In human parasites, no resistance has been convincingly shown yet, although there is an underlying risk due to mass therapy (Geerts *et. al.*, 1997). Attempts have been made to understand the mechanisms of resistance as they are key to the elucidation of the molecular mechanism of action of the drugs in use (Roos, 1997). In general, resistance arises due to 1) an altered drug target site or 2) a reduction in the drug reaching the target site due to increased uptake or drug efflux, or altered drug metabolism. An altered site for example is observed for benzimidazole resistance in *H. contortus*. The sequence encoding the β tubulin gene from benzimidazole (BZ) susceptible and resistant populations showed a single amino acid substitution of a Phe→Tyr at position 200 (Kwa *et. al.*, 1993; Lubega and Prichard, 1991). The function of this polymorphism at amino acid 200 was finally determined using *C. elegans* as an expression system. (Kwa *et. al.*, 1995). A BZ resistant strain of *C. elegans* was transformed with the β tubulin gene isolated from a BZ susceptible *H. contortus* population. Expression of the *H. contortus* gene altered the phenotype of transgenic *C. elegans* from resistant to susceptible.

Resistance to levamisole has been slow to develop in *H. contortus* (Conder *et. al.*, 1991). Among pig helminths, levamisole resistance has been shown exclusively in *Oesophagostomum quadrispinulatum* (Roepstorff and Nansen, 1994). It is not known whether *A. suum* may be a strong candidate for the development of resistance. Inheritance of levamisole resistance is mainly attributed to a single recessive gene or closely linked group of genes located on the X chromosome (Martin and McKenzie, 1990). The mechanism of resistance is thought to be associated with a reduction in the number of nicotinic acetylcholine receptors of nematodes, or a change in their binding characteristics (Lewis *et. al.*, 1980; Sangster *et. al.*, 1988). Although levamisole resistant mutants associated with this receptor have been studied in detail in *C. elegans*, little is understood about it in the parasitic nematodes (Lewis *et. al.*, 1987; Hoekstra *et. al.*, 1997).

Indiscriminate and repeated use of ivermectin has also resulted in resistance in

several field strains of *H. contortus* (Carmichael *et. al.*, 1987; Van Wyk *et. al.*, 1988). Investigation of the mechanisms of ivermectin resistance has been mainly approached through studies in *C. elegans* susceptible to the drug (Schaeffer and Haines, 1989). A direct correlation between the *in vivo* potency of avermectin and its analogues, and their high affinity binding sites was shown in membrane preparations of *C. elegans*. Further, ivermectin was shown to act via glutamate-gated chloride ion channels in nematode cell membranes (Cully *et. al.*, 1994; Arena *et. al.*, 1995). The ivermectin binding sites in *H. contortus* appeared to be similar to that observed in *C. elegans* with respect to affinity for ivermectin and receptor density. Receptor density was the same in both ivermectin susceptible and resistant nematodes, suggesting that target sites for ivermectin binding were not involved in the development of drug resistance (Rohrer *et. al.*, 1994).

1.6.3 Alternative methods of parasite control

The constant increase in anthelminthic resistance of parasites of livestock has led to the search for alternatives for chemotherapeutic control. Resistance to chemicals occurs faster than the rate at which new anti-parasitic drugs are being developed by the pharmaceutical industry. There are also global concerns about the environmental impact of chemical use and the risk of chemical residues in meat and other edible animal products (Gill and Le Jambre, 1996). Control measures such as reduction of livestock density on pastures, or an increase in the degree of stock rotation on to clean pastures by alternating grazing between species such as sheep and cattle have been implemented as grazing management schemes (Strong and Wall, 1990). Biological control methods include the use of nematophagous fungi to trap and destroy larvae in faeces. The fungi produce characteristic traps on their mycelia or produce chemo-attractant and/or chemotoxic substances to nematodes. Following capture of the nematode, the fungus penetrates the worm and destroys it (Waller 1992; 1993). In contrast to chemical control of worms directed exclusively at the parasitic stage within the host, biological means of control focus on the free-living stages on pasture. The most attractive method of helminth control however would be the use of vaccines against these parasites.

Development of vaccines has mainly depended on the identification of novel parasite antigen molecules that elicit a protective immune response (Meeusen, 1996). A

range of candidate vaccine antigens have been isolated from major helminth parasites like *Schistosoma mansoni* and *Brugia malayi* (Waine and McManus, 1997; Selkirk *et. al.*, 1992). However, a better understanding of the immune effector mechanisms involved in the host is required. Helminths survive for years in the infected host by evolving elaborate evasion mechanisms and inducing tolerance to the parasite antigens. A dramatic increase in expansion of the Th lymphocyte subset is noted during infection. Further insight into subset selection and the immune pathways induced will help direct an effective immune response to helminth parasites (Allen and Maizels, 1997).

1.7 Neurotransmitters

There are 18-20 non-peptide molecules suggested to act as classical transmitters. Typically, these are synthesised in the nerve terminal, released following nerve stimulation and act post-synaptically at a specific receptor (Walker *et. al.*, 1996). Acetylcholine (ACh) is a major excitatory transmitter. Two distinct types of ACh receptors are known where either nicotine or muscarine act as an agonist. Nicotinic ACh receptors have four transmembrane domains and are linked to fast acting ligand-gated ion channels. Muscarinic receptors have seven transmembrane domains and are linked to the slow-acting G-protein group of second messengers (Hannan and Hall, 1993). Other major transmitter molecules include amines like 5-hydroxy-tryptamine, and amino acids like L-glutamate, L-aspartate, γ-amino butyric acid (GABA), and glycine (See Figure 1.10). GABA and glutamate act as both excitatory and inhibitory neurotransmitters in nematodes. The distribution and function of a range of transmitter molecules in nematodes have been identified by immunocytochemical localisation and pharmacological methods.

1.7.1 Nematode neurotransmitters

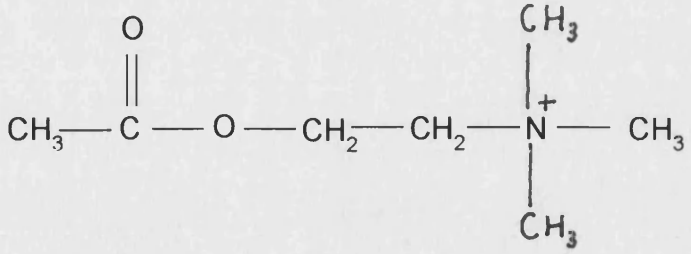
1.7.1.1 Glutamate

Binding sites for L-glutamate in membrane preparations of *C. elegans* were first reported by Schaeffer and Bergstrom (1988) This site was pharmacologically different from vertebrate receptors (Schaeffer *et. al.*, 1990). However, the receptor was

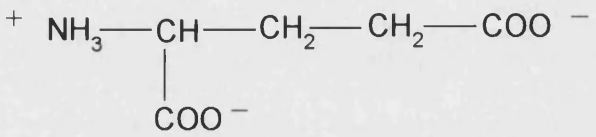
considered an important anthelminthic target when the glutamate antagonist, MK-801 was found to cause paralysing actions in *C. elegans* (Schaeffer *et. al.*, 1989a). Specific binding of MK-801 was observed on membrane preparations at a site pharmacologically different from homologous sites in mammals (Schaeffer *et. al.*, 1989b). Glutamate-gated chloride ion channels were subsequently cloned by Cully *et. al.*, (1994). cDNAs encoding inhibitory glutamate receptor subunits have also been identified in this laboratory in *C. elegans* and *H. contortus* (Laughton *et. al.*, 1994). Recently, glutamate receptors gating chloride channels have been demonstrated in *A. suum* pharyngeal muscle preparations (Martin *et. al.*, 1996). A reversible increase in input conductance associated with a small change in membrane potential was observed upon application of 100μM L-glutamate. A small hyperpolarization of 1mV associated with an input conductance change from 157 μS to a peak of 429 μS, desensitising to 231 μS after 4min was observed. The effect of milbemycinD, an ivermectin-analogue anthelminthic was also examined. An increase in potentiation of glutamate was observed as the effect of milbemycin on input conductance increased.

Currently, interest has grown in the area of glutamate transporters (Fairman *et. al.*, 1995). These transporters have also been classified as transmitter gated channels and contain a chloride channel. In *A. suum*, there is now evidence for a putative electrogenic glutamate transporter (Davis, 1998). It occurs in the hypodermis and in dorsal excitatory DE2 motorneurons receiving glutamatergic post-synaptic potentials. The *A. suum* glutamate transporter exhibits pharmacological profiles similar to the high affinity glutamate binding site identified in *C. elegans* (Schaeffer *et. al.*, 1990; Rohrer *et. al.*, 1990). Partial cDNA clones with significant homology to members of the glutamate transporter gene family have been recently isolated from the nematode *O. volvulus* (Radice and Lustigman, 1996). It is possible that such transporters are represented over a broad range of parasitic nematodes and may serve as potential targets for anthelminthics.

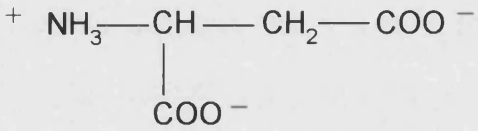
Acetylcholine



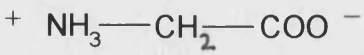
Glutamate



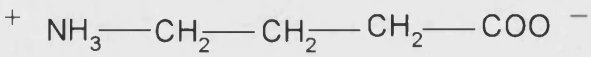
Aspartate



Glycine



GABA



Serotonin

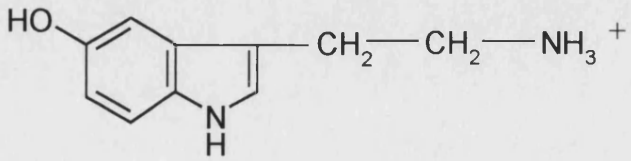


Figure 1.10 Structure of neurotransmitter molecules.

1.7.1.2 GABA

GABA is a major interneuronal transmitter and generally inhibitory in nature. Three pharmacologically and structurally distinct subclasses of GABA receptor types are known in mammals- $GABA_A$, $GABA_B$ and $GABA_C$ (Sieghart, 1995; Sivilotti and Nistri, 1991). The ionotropic $GABA_A$ like receptor subtype is the most widely studied in invertebrates (Walker *et. al.*, 1996). It is a member of a superfamily of ligand-gated ion channel receptors. Binding of GABA to the receptor opens an associated chloride channel. The presence of GABA receptors on *A. suum* muscle was first recognised by the hyperpolarizing effect of bath-applied piperazine and GABA (Del Castillo *et. al.*, 1964 a, c). Piperazine selectively opens GABA-gated Cl^- channels on *A. suum* muscle membranes resulting in flaccid paralysis (Martin, 1993). Voltage clamp studies on the muscle also resulted in a current with reversal potential near –65mV, demonstrating the presence of extrasynaptic receptors over the surface of the muscle cell including the bag region (Martin, 1980; 1982). Pharmacological profiles show that the agonist recognition site of the receptor may resemble the vertebrate $GABA_A$ receptor in activation by compounds like isoguvacine, muscimol or dihydromuscimol (Holden-Dye *et. al.*, 1988). However, it differs from the vertebrate $GABA_A$ receptor in not being blocked by the definitive competitive receptor antagonists, bicuculline and picrotoxin (Wann, 1987; Holden-Dye *et. al.*, 1988).

GABA-like immunoreactivity has been localised in the inhibitory motorneurons of *A. suum* (Johnson and Stretton, 1987). Activation of these neurons elicits inhibitory synaptic potentials in the muscle cells. In *C. elegans*, Mc Intire *et. al.*, (1993b) detected 26 GABA-immunoreactive neurons present within the entire nervous system. Using laser ablation and genetic techniques GABA was shown to have both inhibitory and excitatory roles. The 26 GABAergic cells constitute the 6 DD, 13 VD, 4 RME, 1 AVL, 1 DVB and 1 RIS neurons. The DD and VD motor neurons enable positive feedback and allow the body to bend. The release of GABA results in relaxation of the dorsal and ventral muscles of the animal. The GABAergic RME neurons control the extent to which the head deflects during foraging. The AVL and DVB motor neurons control the extent to which the enteric muscles contract during defecation. Several mutants defective in GABA-mediated behaviours have also been identified. Thus, gene *unc-25* is required for GABA expression, *unc-30* for differentiation of a specific type of neuron, *unc-46* and *unc-47* in

normal GABA release and for normal adult male sphincter relaxation, and *unc-49* in exerting a postsynaptic inhibitory effect of GABA on the body muscles (McIntire, 1993a; Reiner and Thomas, 1995).

1.7.1.3 Acetylcholine

Initial studies showing the presence of ACh receptors in *A. suum* were carried out on muscle strips, followed by the demonstration of membrane depolarization in single muscle cells, and the presence of cholinesterase in the nervous system (Baldwin and Moyle 1949; Norton and De Beer, 1957; Del Castillo 1964b; Knowles and Casida; 1966). The agonist profile of the ACh receptor on the muscle cell shows that it is nicotinic as it is blocked by tubocurarine, and not atropine (Holden-Dye and Walker, 1991). But further classification into ganglionic or neuromuscular nicotinic ACh does not appear possible. For example, mecamylamine, a ganglionic nicotinic antagonist and benzoquinonium, a neuromuscular antagonist are both potent antagonists on the *Ascaris* nicotinic ACh receptor (Martin *et. al.*, 1996). The receptor is the primary site of action of anthelminthics levamisole, pyrantel and morantel (Aubry *et. al.*, 1970; Harrow and Gration, 1985). These are more potent agonists at the *A. suum* ACh receptor than at vertebrate nicotinic ACh receptors, and cause depolarization and spastic paralysis of the nematode without a significant action on the host muscle (Robertson and Martin, 1993; Robertson *et. al.*, 1994; Evans and Martin, 1996). The pharmacologies of both the vertebrate and the *A. suum* nicotinic ACh receptor have been extensively studied by Colquhoun *et. al.* (1991). ACh receptors have been demonstrated on *A. suum* motor neurones by specific intracellular recordings from commissural axons, although their physiology and pharmacology needs further understanding (Stretton *et. al.*, 1992). Motor neurons DE1, DE2, DI and VI have ACh receptors sensitive to 100μM tubocurarine (Segerberg and Stretton, 1993). However, DE1 alone is found to possess receptors activated by 100μM N-methyl scopolamine, a classical muscarinic antagonist, the activation not being blocked by *d*-tubocurarine. Thus the cell types appear to lack identical cholinergic pharmacology.

In *C. elegans*, three genes *lev-1*, *unc-38* and *unc-29* involved in levamisole resistance have been shown to encode for subunits for nicotinic ion-channels (Fleming *et.*

al., 1997). When co-expressed in *Xenopus* oocytes, they resulted in functional channels gated by levamisole. However, other subunits like *ACh1* (also referred to as Ce21) expressed as a homo-oligomer in *Xenopus* oocytes producing channels sensitive to nicotine, but not levamisole have been reported (Ballivet *et. al.*, 1996).

1.7.1.4 5-hydroxytryptamine (5-HT, Serotonin)

5-HT plays an important role in controlling behaviour and physiological processes such as feeding and locomotion in nematodes. In *A. suum*, 5-HT stimulates the pharynx and is involved in glycolytic metabolism (Donahue *et. al.*, 1981; Martin *et. al.*, 1988). Through an *in vitro* pharmacological approach, Brownlee *et. al.* (1995) have shown that although the pharynx does not contract spontaneously, it can be stimulated by 5-HT (10μM0-1mM) at a frequency of 0.5Hz. Immunoreactivity to serotonin is observed in nerve fibres and nerve plexuses within the enteric nervous system of *A. suum* (Brownlee *et. al.*, 1994a). The distribution of serotonin-like immunoreactivity is observed in two motor neurones in the pharynx of both sexes that are likely to be neurosecretory in nature, and five neurons in the adult male tails (Johnson *et. al.* 1996). The role of serotonin in locomotion has also been demonstrated by injecting it into *A. suum* (Reinitz and Stretton; 1996). This results in immediate paralysis, an increase in body length, and decreased number of propagating body waves. In *C. elegans*, 5-HT plays an important role in locomotion, feeding, male-mating, defecation, and egg-laying. (Horvitz *et. al.*, 1982; Avery and Horvitz, 1990; Loer and Kenyon, 1993; Johnson *et. al.*, 1996; Desai *et. al.*, 1988; Desai and Horvitz, 1989). However, some differences in associated phenotypes of 5-HT deficient mutants suggest that 5-HT alone is not sufficient for egg-laying and requires the combined action of acetylcholine (Weinshenker *et. al.*, 1995). Differences in 5-HT immunoreactivity have been observed between *A. suum* and *C. elegans* (Johnson *et. al.*, 1996). For example, a pair of HSN-like neurons (hermaphrodite specific neurons) that label in *C. elegans*, fail to in *A. suum*. In addition, 5-HT immunoreactivity is not observed in certain cells, homologues of which get stained although weakly, in *C. elegans*. The significance of such differences is still unclear.

1.7.2 Neuropepetides

Neuroactive peptides are widely represented in the nematode nervous system (Geary *et. al.*, 1995). Neuropeptides in various nematode species were identified using antisera to localize immunoreactivity against the peptide Phe-Met-Arg-Phe-NH2 (FMRFamide), first isolated from the mollusc *Macrocallista nimbosa* (Price and Greenberg, 1977). The first nematode neuropeptide (Lys-Asn-Glu-Phe-Ile-Arg-Phe-NH2 or KNEFIRF amide), AF1 (*Ascaris* FMRF-amide-like peptide 1) was identified from *A. suum* (Cowden *et. al.*, 1989). Several FMRF-amide related peptides (FARPs), AF1-11 have subsequently been isolated from *A. suum* and other nematode species including *C. elegans* (CF1-7) and *Panagrellus redivivus* (PF1-4). The FARP, AF2 (Lys-His-Glu-Tyr-Leu-Arg-Phe-NH2) appears to be the most common (Maule *et. al.*, 1996a). Although the FARPs are structurally closely related, even small changes in their peptide sequence such as a single amino acid affect the mechanism of action induced by ligand-receptor interaction. For example, AF1 is mainly excitatory on the body wall muscle while the PF4, KPNFIRFamide is inhibitory.

Functionally, several peptides are more potent than the 'classical neurotransmitters' acetylcholine and GABA. Neuroactive peptides also seem to act to modify the nematode physiological processes either within the central ganglia, on the nerve terminals of motor and sensory neurons, or directly on effector organs such as the pharyngeal or somatic body wall muscles (Brownlee *et. al.*, 1996). The peptides known so far are found to interact both presynaptically and postsynaptically at the neuromuscular junction. This is likely to exert its effect on the nematode locomotory behaviour.

Peptides in general appear to predominate the nematode neural signalling systems (Maule *et. al.*, 1996b). As the peptide sequences vary between species and phyla, these appear to be attractive targets for selective toxicity to the parasite and not its host. However, a better understanding of the occurrence of endogenous peptides and their function is still required.

1.8 Nematode receptors

Two major modes of action of anthelminthics have been exploited so far- drugs acting on parasite membrane ion channels which have a rapid therapeutic effect, and those

acting on biochemical target sites. The family of ligand gated ion channels with target sites for the action of anthelminthic drugs consists of nicotinic ACh receptors, GABA receptors, and inhibitory glutamate receptors (IGluRs). Several receptor subunits from this family have been cloned from nematodes.

1.8.1 Structure

A major family of multimeric membrane-spanning proteins, the ligand-gated ion channels includes the nicotinic ACh, $GABA_A$, glycine and 5-HT_3 receptors (Bertrand and Changeux, 1995; Darlison and Albrecht, 1995; Kuhse *et. al.*, 1995; Maricq *et. al.*, 1991). Recent cloning of some of the invertebrate glutamate-gated chloride (GluCl) ion channels suggests that these are also members of the same family. No GluCl ion channels have been reported in vertebrates (Cully *et. al.*, 1994). The structure of the receptors of this family have mainly been based on comparisons with the muscle nACh receptor of the torpedo electric organ. Its structural analysis has been definitively established at 9Å resolution (Unwin, 1993). The same pentameric structure has also been established for the native $GABA_A$, 5-HT_3 and glycine using electron microscope image analysis of single receptor particles (Nayeem *et. al.*, 1994; Boess *et. al.*, 1995). The receptor operated transmembrane ion-channel complex is a heteropentamer. It is composed of five subunits which include two α, one β, one γ and one δ subunit, presumably arranged pseudo-symmetrically around a central channel forming a cation conducting pathway. The channel is suggested to open when ACh binds both α subunits possessing distinct conformations (Unwin, 1996). The channel opens transiently to release ACh from the nerve terminal into the synaptic cleft.

Each subunit consists of an extracellular NH_2-terminal half with N-linked glycosylation sites. A pair of cysteines spaced at a gap of fifteen residues, and predicted to form a Cys-Cys loop, occurs in the extracellular domain. The NH_2-terminal end is followed by four putative hydrophobic transmembrane spanning domains, TM 1-4 and a short COOH terminus. The α-helical structure of the TM2 domain has also been shown in the rat α1 $GABA_A$ receptor subunit (Xu and Akabas, 1996). TM 1-TM 3 are closely spaced. The pore of the ion conducting channel is lined by residues in the TM 2 domain (Sakmann, 1992; Karlin, 1993). A schematic outline of a receptor subunit is shown in

Figure 1.11. Three negatively charged rings occur in this region- an extracellular ring lined by glutamate and aspartate, a narrow middle ring lined with glutamate, and a third inner cytoplasmic ring lined with glutamate and aspartate. The middle ring, believed to form the selectivity filter of the ion channel, allows only positively charged cations through the ion pore (Revah *et. al.*, 1990). The nACh receptor is thus able to conduct sodium, potassium, and calcium to a lesser extent through the pore. GABA and glycine receptors on the other hand have a high positive charge around the pore region.

A large cytoplasmic intracellular loop domain with hydrophilic amino acids occurs between TM 3 and TM 4 and is highly variable in sequence. Some of the cytoplasmic loops contain consensus sequences for phosphorylation by various protein kinases involved in regulatory mechanisms. For example, two alternatively spliced forms of the mammalian $\gamma 2$ $GABA_A$ receptor subunit exist in bovine and mouse brain. These forms vary only in their intracellular loop region which includes a protein kinase C phosphorylation site (Harvey *et. al.*, 1994; Whiting *et. al.*, 1990). This suggests that the receptors containing different forms of the $\gamma 2$ subunit may be differentially regulated by PKC.

The functional domains of $GABA_A$ receptors have been examined by site directed mutagenesis and a model proposed for the agonist binding site in transmitter-gated ion channels (Smith and Olsen, 1995). Two α subunit regions beginning at Thr60 and Thr95 are likely to be involved in GABA and benzodiazepine binding sites respectively. A common motif TXDXFF occurs in these two regions. The Thr residue near the NH_2 terminal of these two regions is conserved in $GABA_A$ receptors and other transmitter-gated ion channel receptor subunits. The His 101 residue confers the benzodiazepine binding. Mutation of this residue to Arg results in a subunit insensitive to agonists. Interestingly, a sequence alignment of the invertebrate GluCl receptors shows that the first TXDXFF motif is absent and the second is substituted by a highly conserved PDTFF motif. Highly conserved among all members of the superfamily are the Trp69 and Trp 94 residues of the $GABA_A$ receptor. The region formed by these two conserved residues could correspond to a functional domain performing a similar role among all members of the superfamily. For example the energy of ligand binding alone is probably not sufficient to cause a conformational shift resulting in channel opening, a concerted movement of a larger motif may be necessary for channel function. It is suggested that the conserved Trp

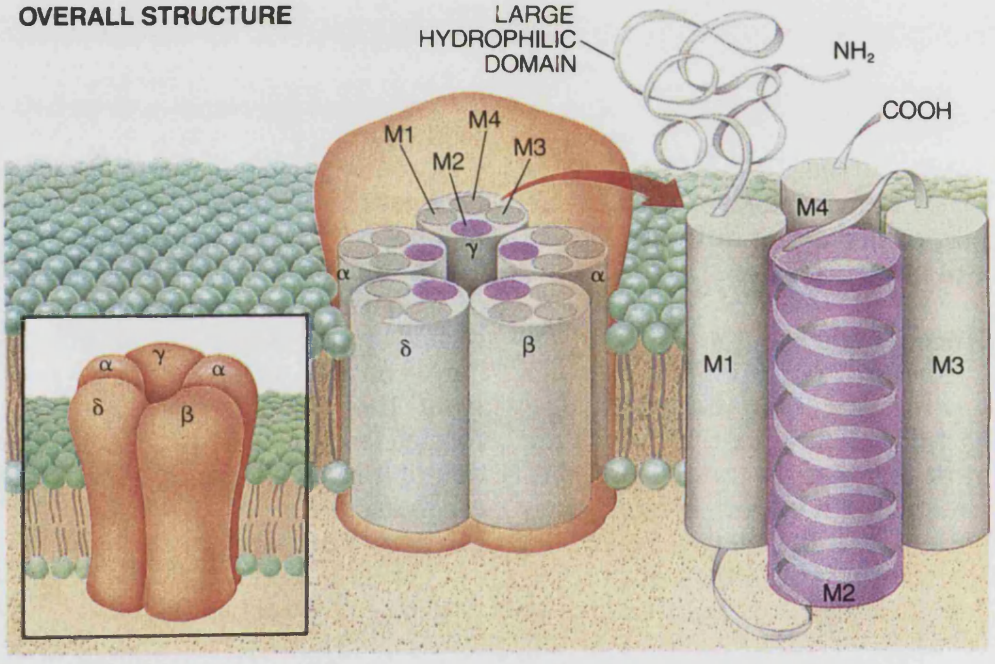


Figure 1.11 Schematic of a receptor subunit.

residues could participate in translation of this essential motion due to their size and hydrophobicity, causing them to be concentrated together in the interior of the protein. This could act as a structural anchor for transmission of this energy to other parts of the receptor protein.

* Figure 1.11 adapted from 'Chemical signaling in the brain' by Jean Pierre Changeux, Scientific American, November 1993.

1.8.2 Receptor heterogeneity

Subunit heterogeneity has made it difficult to determine the composition and stoichiometry of receptor subunits forming the ion channel (Green and Millar, 1995). Although there is direct evidence only for the *Torpedo* muscle nACh receptor as a pentamer of $\alpha_2\beta\gamma\delta$, many different combinations of the receptor subunits are possible. Heterologous expression of the subunit combinations $\alpha\gamma$, $\alpha\delta$, $\alpha\beta\gamma$, $\alpha\beta\delta$ and $\alpha\gamma\delta$ have resulted in functional receptors but only the $\alpha_2\beta\gamma\delta$ receptors in *Torpedo* are found to assemble *in vivo* (Barnard, 1992). The vertebrate neuronal nicotinic ACh receptors on the other hand constitute only α and β subunits (McGhee and Role, 1995). Several isoforms ($\alpha_{2\text{-}9}$, $\beta_{2\text{-}4}$) possibly assembling as pentamers have been identified (Cooper *et. al.*, 1991). Multiple isoforms of the $GABA_A$ receptor subunits, α, β, γ, δ and ρ are also known to occur (α1-6, β1-3, γ1-3 and ρ1-2) (Stephenson, 1995; Whiting *et. al.*, 1995). Some of the $GABA_A$ receptor genes undergo alternative splicing, for example the γ2 subunit occurs in two forms- $\gamma 2_{\text{short}}$ and $\gamma 2_{\text{long}}$. The functional significance of such a wide variety of receptor subtypes is, however, not very clear. It is possible that subunit combinations influence important channel modulating properties via intracellular mechanisms such as activation of different protein kinases (Krishek *et. al.*, 1994).

Among invertebrate $GABA_A$ receptors, a fair number of subunits have been cloned. In general, the sequences of these subunits show homology to the vertebrate α or β $GABA_A$ type of receptors. In *Drosophila*, these include the RDL subunit (resistant to dieldrin), a β-subunit, and a GRD subunit (GABA-A-Glycine-like subunit *Drosophila*) (ffrench-Constant *et. al.*, 1991; Henderson *et. al.*, 1993; Harvey *et. al.*, 1994). The RDL gene resembles a mammalian glycine subunit in sequence but the receptor it encodes is insensitive to glycine when expressed in *Xenopus* oocytes. It is however, activated by GABA and muscimol, confirming it to be a GABA receptor. All three subunits have a conserved TTVLTMTT sequence located in the second transmembrane domain suggesting that they form anion-selective channels. A homologue of the RDL receptor subunit has been cloned from the mosquito *Aedes aegypti* (Shotkoski *et. al.*, 1994). Other $GABA_A$ like receptor subunits that have been cloned include three from *Lymnaea stagnalis* (Harvey *et. al.*, 1991; Hutton *et. al.*, 1993; Darlison *et. al.*, 1994) and one from *H. contortus* (Laughton *et. al.*, 1994).

1.8.3 Nematode inhibitory glutamate receptors

Glutamate-gated chloride (GluCl) channels are found in nematodes, insects, crustaceans and molluscs. This was first reported from locust, *Schistocerca gregaria* leg muscle preparations (Cull-Candy and Usherwood, 1973). The avermectins were subsequently shown to activate GABA-independent chloride conductance in the leg (Duce and Scott, 1985a). Activation of H-receptors of locust leg muscle were shown to result in the avermectin-sensitive current (Duce and Scott, 1985b). Among nematodes, the expression cloning of GluCl channels from *C. elegans* in *Xenopus* oocytes opened a series of studies on the mechanism of action of the avermectin anthelminthics (Cully *et. al.*, 1994). Two subunits GluClα (now called GluClα1) and GluClβ, possibly assembling as a pentamer have been cloned. The predicted amino acid sequences of the two subunits show 45% identity with each other. The sequences also possess motifs common to GABA and glycine ionotropic receptor subunits that are part of the Cys-loop family. The individual subunits GluClα1 or GluClβ, express functional homomeric channels. Homomeric GluClβ channels are directly gated by glutamate, demonstrating that the GluClβ subunit contains all the determinants for ligand binding and coupling to channel gating. Homomeric GluClα1 channels are insensitive to glutamate, but are directly activated by ivermectin phosphate. Coexpression of the two subunits resulted in ivermectin-potentiated heteromeric GluCl channels. The EC-50 for ivermectin with GluClα1 was 140nM, and 190nM upon coexpression of both subunits. EC-50 for glutamate against GluClβ was 380μM, and 1360μM upon coexpression. Ivermectin potentiates glutamate-induced chloride ion currents at nanomolar concentrations in oocytes injected with GluClα1 and GluClβ, but not in the presence of GluClβ alone. Picrotoxin is a weak antagonist and the channels are activated with ibotenate but not GABA, glycine, or a host of glutamate-gated cation and ligand gated chloride channel agonists. The major determinants of glutamate binding are present on the GluClα N-terminal (Etter *et. al.*, 1996). This was determined by constructing a chimera of GluClα and GluClβ in the region of the extracellular domain of GluClα. A glutamate sensitive current with an EC50 of 530 +/- 90nM was observed when oocytes were injected with RNA from this construct. In addition, a single amino acid substitution of a naturally occurring threonine in the TM2 region of GluClα subunit to a proline, glycine, or alanine enabled glutamate gating of GlClα channels, suggesting that subtle conformational

changes were sufficient to result in changes in channel gating.

Phylogenetic analysis of the protein sequences of the GluCl genes show that these bear high amino acid identity with each other and are distinct from $GABA_A$ and glycine receptor subunits (Figure 1.12). A table of the percent amino acid identities between these subunits is also shown in Table 1.2. Other cDNAs encoding GluCl channels have been isolated from *C. elegans* (GluClα2A, Gluclα2B, Ce-GBR-2A, Ce-GBR-2B, GluClX, C27H5.8), *Drosophila melanogaster* (DrosGluClα), and filarial nematodes *Dirofilaria immitis* and *Onchocerca volvulus* (GluClX) (Cully *et. al.*, 1996a). The DrosGluClα subunit shares 48% amino acid identity with the *C. elegans* GluClα and β subunits (Cully *et. al.*, 1996b). The DrosGluClα subunit exhibits pharmacological characteristics similar to the H-receptor GluCl channels reported from locust leg muscle, but differs from the GluCl α and β receptors in *C. elegans*. For example, DrosGluClα alone is sufficient to gate both ivermectin and glutamate, unlike *C. elegans* wherein both the α and β subunits are required. It rapidly and completely desensitises in the continued presence of glutamate, unlike the slow and incomplete desensitisation of the nematode channel (Cully *et. al.*, 1994). Also, the DrosGluClα channel is less sensitive to ivermectin potentiation of the glutamate response unlike the *C. elegans* α and β channels.

Genes encoding putative GluCl-related subunits in *C. elegans* have been identified from the genome sequencing project which has involved extensive genetic characterisation with ordered physical maps (Wilson *et. al.*, 1994). A full-length cDNA sequence corresponding to cosmid C27H5.8 (previously called C27H5.5) encoding a ligand gated ion channel and a putative member of the GluCl channel family has thus been isolated by Cully *et. al.*, (1996b). Another predicted cDNA sequence corresponding to cosmid ZC317.3 showing homology to sequences in the GluCl family is currently being amplified using the PCR strategy in this laboratory. Mutations in the *C. elegans* GluCls and related genes may result in a variety of phenotypes such as defects in coordination of movement (*unc*), in eating behaviour (*eat*) and in egg laying (*egl*). Wild-type worms treated with ivermectin which interact with the GluCl channel exhibit alterations in these phenotypes. For instance, GluCls associated with an ivermectin resistant mutant, *avr-15*, showing eating behaviour defects have been identified using YAC clones by Avery *et. al.*, (1997). Other *C. elegans* cosmid clones that may encode for putative GluCl-related genes owing to their sequence homology and need further investigation have been listed in Table 1.3.

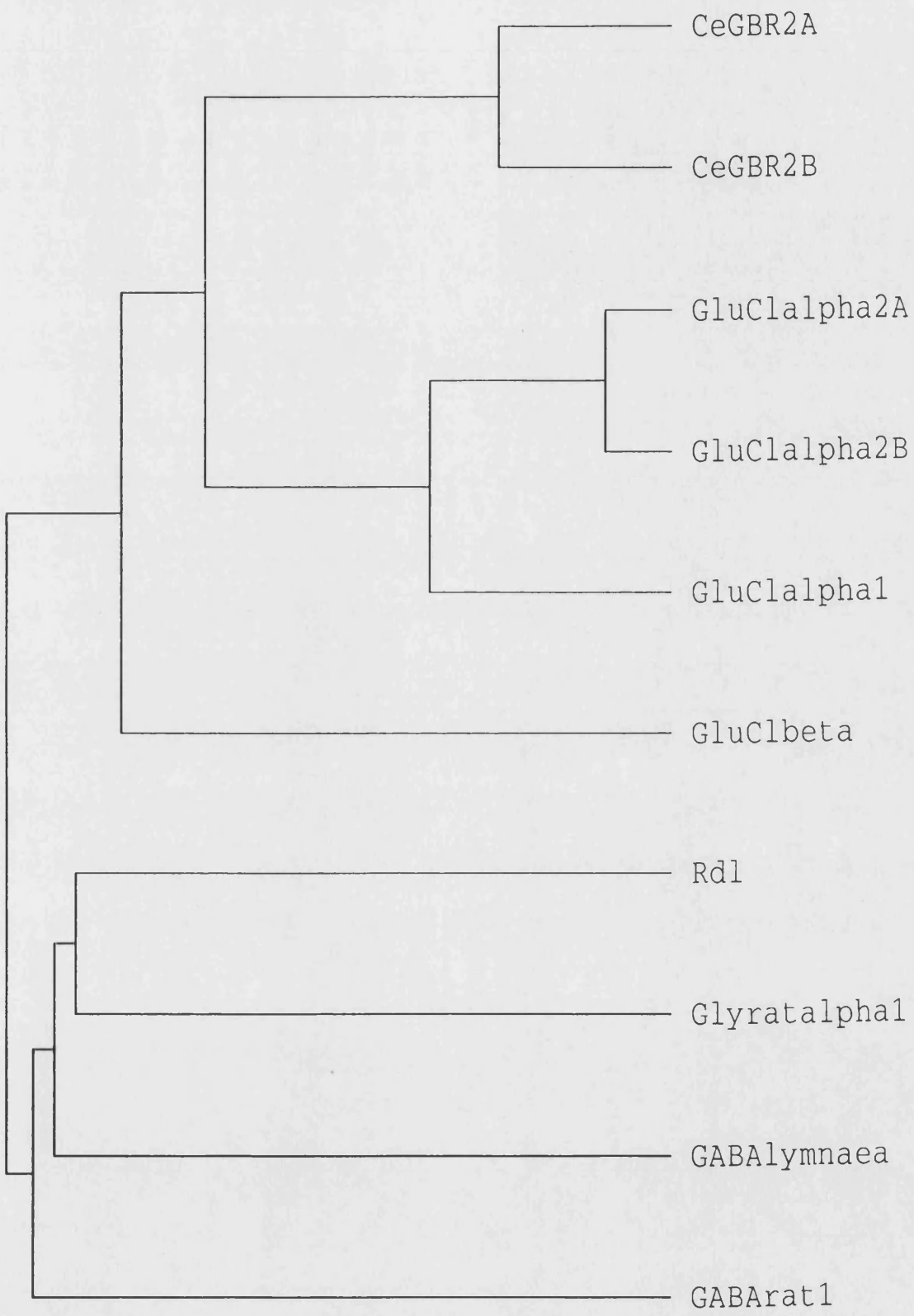


Figure 1.12 Tree showing sequence relationships between GABA, glycine and glutamate receptor subunits. The tree was generated using the 'PILEUP' program in GCG (Devereux *et. al.*, 1984).

1 Ce-GBR-2B
2 Ce-GBR-2A
3 GuClα2B
4 GluClα2A
5 GluClα1
6 GluClβ
7 Glyratα1
8 Rdl
9 GABAlymnaea
10 GABArat1

	2	3	4	5	6	7	8	9	10
1	82	53	52	53	47	39	35	30	34
2		57	57	56	49	42	39	32	37
3			96	75	48	40	36	33	33
4				74	48	40	36	33	33
5					47	39	31	32	32
6						39	34	30	30
7							37	38	34
8								35	32
9									35

Table 1.2 Percent amino acid identity between the GABA, glycine and glutamate receptor subunits. The table was generated using the GCG program DISTANCES and subtracting the values obtained from 100 (Devereux *et. al.*, 1987).

Subunit	Source	Accession No.	Reference
GluClα1	*C. elegans*	U14524	Cully *et. al.*, 1994
GluClβ	*C. elegans*	U14525	Cully *et. al.*, 1994
GluClX	*C. elegans*	U59743	Cully *et. al.*, 1996
Ce-GBR-2A	*C. elegans*	U40573	Laughton *et. al.*, 1997
Ce-GBR-2B	*C. elegans*	U41113	Laughton *et. al.*, 1997
GluClα2A	*C. elegans*	AJ000538	Dent *et. al.*, 1997
GluClα2B	*C. elegans*	AJ000537	Dent *et. al.*, 1997
C27H5.8	*C. elegans*	U14635	Cully *et. al.*, 1996
ZC317.3	*C. elegans*	U64840	ACeDB
GluClX	*Dirofilaria immitis*	U59745	Cully *et. al.*, 1996a
OvGluCl	*Onchocerca volvulus*	U59745	Cully *et. al.*, 1996a
DrosGluCl	*Drosophila melanogaster*	U58776	Cully *et. al.*, 1996b

Table 1.3 List of invertebrate genes encoding glutamate-gated chloride (GluCl) ion channels and GluCl-related genes.

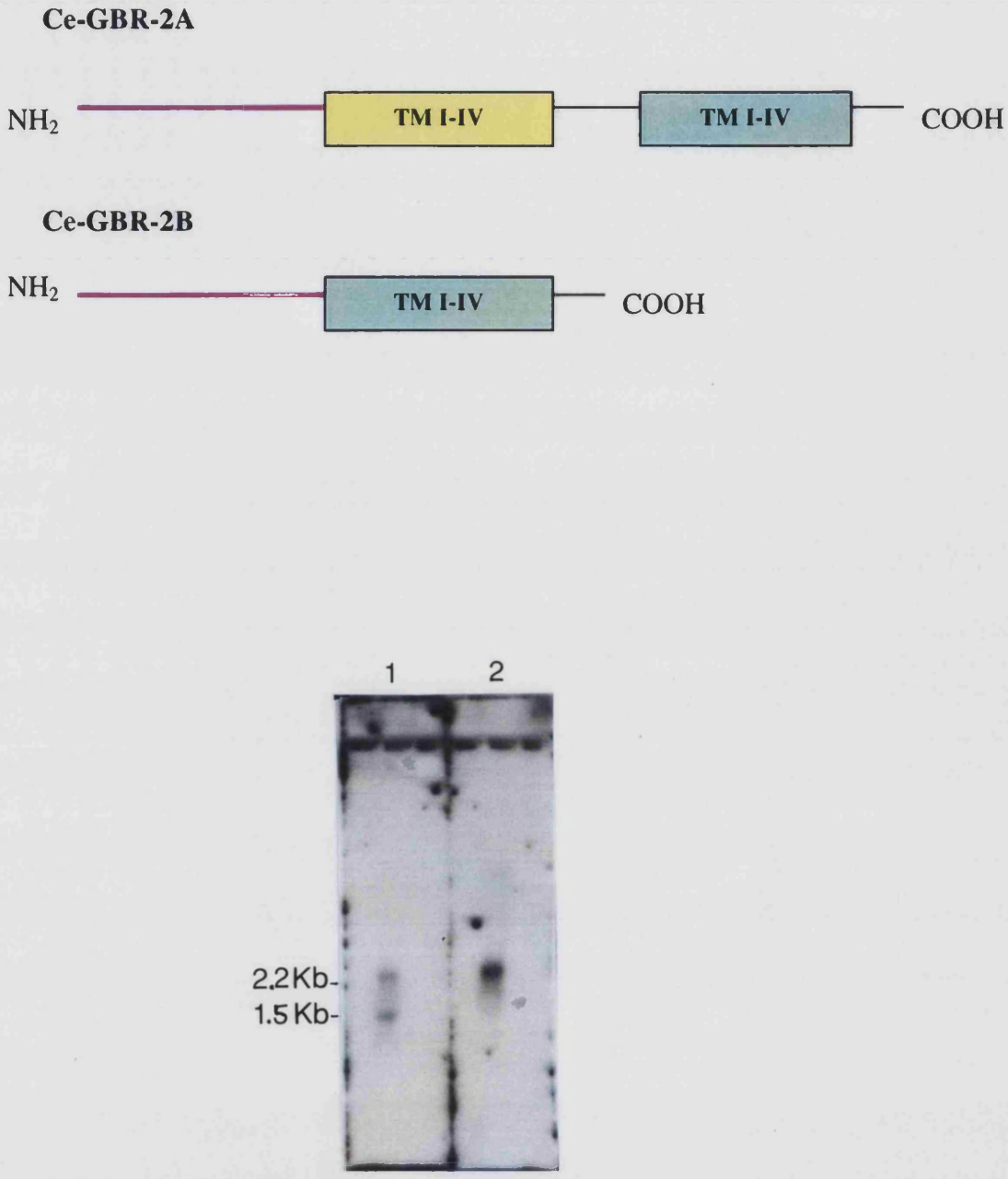


Figure 1.13 **(A)** Schematic of the products obtained as a result of alternative splicing of the *gbr-2* gene in *C. elegans*. Common NH_2-terminal domain is shown in purple. **(B)** Northern blot of *C. elegans* mRNA probed with (1) *gbr-2A* and (2) *gbr-2B* specific sequences (from Laughton *et. al.*, 1997).

1.8.4 Alternatively spliced nematode GluCl channels

The first report of an alternatively spliced gene in *C. elegans* encoding inhibitory amino acid receptor subunits came from this laboratory. PCR amplifications on *C. elegans* cDNA using oligonucleotide primers matching conserved sequences found in invertebrate $GABA_A$ receptors resulted in the amplification of several partial cDNAs. These showed high homology to $GABA_A$ and glycine receptor subunits. RACE-PCR amplifications of one of the cDNAs denoted *gbr-2A* (**GABA** family of **r**eceptor subunits) generated a full-length 2.15Kb long sequence (Laughton *et. al.*, 1997). This encodes for a protein of 416 amino acids. The *gbr-2A* cDNA clone includes a large 3′ untranslated region (UTR) of 876bp, within which is present a possible open reading frame (ORF). The ORF encodes for the C-terminal portion with four transmembrane domains, TM I-TM IV of a second receptor subunit, GBR-2B. Thus both GBR-2A and GBR-2B are alternatively spliced receptor subunits with common NH_2-terminal domains but differing COOH-terminal domains (Figure 1.11). The presence of two mRNA transcripts *gbr-2a* and *gbr-2b* was also confirmed using Northern blots (Laughton *et. al.*, 1997). Sequences of the two receptor subunits showed highest identity to the GluCl subunits isolated from *C. elegans*.

The *gbr-2* gene has recently been shown to map very close to the *avr-14* gene for resistance to ivermectin in *C. elegans* by Dent and Avery (1998). *avr-14* maps close to another gene, *avr-15* which also confers resistance to ivermectin and encodes for a GluCl channel subunit. Substantial resistance to ivermectin was observed in worms with mutations in both *avr-14* and *avr-15*; *avr-14* single mutants exhibit no resistance. Alleles of *avr-14* were therefore isolated by mutating *avr-15* worms and selecting for survival from egg to adulthood in the presence of 10ng/ml ivermectin. All the isolated alleles of *avr-14* mapped to the *gbr-2* gene. This was confirmed by rescuing ivermectin sensitivity of an *avr-14*; *avr-15* double with a cosmid containing *gbr-2*. *Xenopus* oocyte expression of the *avr-14* transcripts showed that GBR-2B responded to both ivermectin and glutamate while GBR-2A did not respond to either (Dent and Avery, *pers. comm.*).

The ivermectin resistance strain, *avr-15* is shown to encode a GluClα2 subunit, (Dent *et. al.*, 1997). A PCR generated GluClα (isolated by Cully *et. al.*, 1994) genomic DNA clone was used to probe a YAC grid. This resulted in strong hybridization to YACs representing the map position of GluClα, and weakly to YACs mapping to the cosmid

K10B8. The latter was found to encode for two alternatively spliced cDNAs, GluClα2A (2.2Kb) and GluClα2B (1.7 Kb). The putative open reading frame bears 85% and 54% amino acid identity to GluClα and GluClβ subunits respectively. GluClα2A responds to both glutamate and ivermectin. While the ivermectin induced response partially desensitizes, the glutamate response desensitizes rapidly. The alternatively spliced transcripts share the exons encoding the putative transmembrane and ligand binding domains. The non-shared extracellular domains include 202 and 23 amino acids of the 2A and 2B subunits respectively. The large extracellular domain in GluClα2A is unique to all the known ligand gated chloride channel subunits and its role is not clear (See Appendix II). It does not appear to affect gating by glutamate or ivermectin as both GluClα2A and 2B respond similarly to these ligands.

1.8.5 Localisation of the GluCl receptor subunits

The GluClβ subunit in *C. elegans* was characterised by LacZ reporter gene constructs and shown to be expressed in the pharyngeal muscle (Laughton *et. al.*, 1997). All developmental stages in *C. elegans* express the GluClβ subunit in the three pharyngeal m4 muscles cells, constituting the metacorpus. The muscle cells are required for pharyngeal pumping and feeding. Their relaxation is triggered by the fast inhibitory M3 motor neurone which generates inhibitory post synaptic potentials (IPSPs) in the contracted pharyngeal muscle (Raizen and Avery, 1994). Localization of the avermectin receptor, GluClβ, to the pharynx thus suggests that this may serve as a potential anthelminthic target. Pharyngeal paralysis would block feeding and eventually result in starvation and death. The location of the GluClα subunit is yet to be determined.

The glutamatergic nature of the M3 neuron in *C. elegans* was confirmed recently by Dent *et. al.*, (1997). Glutamate mimics the effect of the M3 neuron when applied on exposed pharynx, shortening muscle contraction during a pump. In addition, the *avr-15* mutant lacking M3-generated IPSPs was shown to be insensitive to iontophoretically applied glutamate. A GFP (Green Fluorescent Protein) fusion with the region encoding the first three exons of the Gluclα2A subunit expresed in all the m4 muscles of the metacorpus and the m5 muscles of the isthmus of the pharynx respectively. The M3 neuron synapses onto these muscles. Staining was also observed in the RMED, RMEV

and RMG neurons in the head. The expression of the GluClα2A subunit in the pharyngeal muscle and the motor neurons suggests that ivermectin may interfere with locomotion via its interaction with receptors expressed on neurons. GFP fusion with the first six exons of *avr-14* however are expressed in the nerve ring and two neurons in the tail, but not in the pharynx (Avery, *pers. comm.*). Attempts to localise other putative GluCl receptor subunits ZC317.3 and C27H5.8 by GFP fusions are in progress in this laboratory.

1.8.6 GluCl receptor subunits in *H. contortus* and *A. suum*

A series of partial receptor subunits, HG1-HG5 were amplified in this laboratory from a mixed egg population of *H. contortus* (Laughton *et.al.*, 1994). Degenerate oligonucleotide primers were designed to aligned vertebrate and invertebrate $GABA_A$ and glycine receptors showing high amino acid conservation especially in potential ligand-binding and channel forming sites. The primers were made to conserved motifs within the extracellular and the first transmembrane domain, TM I of these receptors (See Appendix I). This resulted in amplification of a product of about 0.45 Kb. Full length amplifications of the partial receptor subunits were carried out using the RACE-PCR procedure (Frohman *et. al.*, 1989; Towner and Gartner, 1992). The presence of a common spliced leader sequence, SL1, *trans*-spliced on the 5′ end of most nematode mRNAs has been exploited for use as the sense primer in the PCR-cloning methods (Bektesh *et. al.*, 1988; Blumenthal and Thomas, 1988). The cDNA *HG1* encoded an inhibitory amino acid receptor subunit and showed a small response to 1mM glycine. Full length cDNAs of *HG4* and *HG5* have also been amplified, showing high sequence identity with the *C. elegans* GluClβ and GluClα subunits respectively (Delany. N, *pers. comm.*). The partial subunit cDNA, *HG2/3* shared high identity with the GBR-2A/2B subunits (>65%) of the GluCl family in *C. elegans*. The *HG2/3* cDNA also contained a long 3′ untranslated region, with a unique set of four transmembrane domains, suggesting that this may be an orthologue of the *Ce-gbr-2* gene. The missing 5′ terminal sequence of *HG2/3* (*HG2/3F*) was determined by Skinner. T., (Thesis, 1997) by the RACE-PCR method using gene specific antisense primers and sense primer SL1. PCR amplification on *A. suum* cDNA using the same degenerate primers also resulted in a 0.45 Kb fragment, *Asg2*, probably encoding a putative receptor subunit (Laughton, *pers. comm.*).

1.9 Aim

Nematodes *H. contortus* and *A. suum* were chosen as model parasitic organisms for the study of receptor subunits, acting as potential anthelminthic targets. The first half of the project was aimed at obtaining the full length sequence of the partially cloned *HG2/3* cDNA from *H. contortus*. The partial HG2/3 cDNA exhibits high identity to the alternatively spliced *C. elegans gbr-2* gene. Comparisons between *HG2/3* and *Ce-gbr-2* sequences at the cDNA and the genomic level, and further analysis of the expression of the possibly alternatively spliced *HG2/3* cDNA could therefore prove to be useful. Finally, the *HG2/3* sequence was to be examined in ivermectin resistant isolates of *H. contortus* and compared with the sequence in the ivermectin susceptible isolate for any possible differences.

The second half of the project involved sequencing the putative partial receptor subunit cDNA *Asg2* amplified from *A. suum* and obtaining the full-length sequence of the same.

The subunits obtained thereof were to be localised in *H. contortus* and *A. suum*. Results of the localisation studies in progress in *C. elegans* for characterisation of the Ce-GBR-2A/2B receptor subunits (Horoszok, L. *pers. comm.*) may be employed for further analysis of orthologous subunits in the parasitic nematodes. The expression patterns of HG2/3 and ASG2 were to be examined.

2. MATERIALS AND METHODS

(A) Materials

2.1 General Laboratory Reagents

REAGENTS AND MATERIALS	SUPPLIER
Acrylamide	Flowgen Instruments Ltd., Kent, UK.
Agarose (Hi-Pure™ Low EEO)	Bio/Gene Limited, Cambridge, UK.
Anti Rabbit-IgG (TRIT-C conjugate)	Sigma Chemical Company Ltd., Dorset, UK
Bacto-agar	Difco, Surrey, UK.
Bovine Serum Albumin	Sigma
Calf intestinal phosphatase	Boehringer-Mannheim Ltd, Sussex, UK.
Chemicals	BDH Dorset, UK; Sigma
CL6B resin	Pharmacia
Collagenase	Sigma
Cryotubes	Nunclon (NUNC), Gibco BRL Renfrewshire, UK.
Cyanogen bromide activated Sepharose™	Pharmacia Biotech, Hertfordshire,UK
DAKO Pen for immunocytochemistry	DAKO A/S, DK-2600, Denmark
DEPC	Sigma
Dialysis tubing	Medicell International Ltd., London, UK.
DNA Polymerase I	New England Biolabs Inc., (NEB), Hertfordshire,UK.
dNTPs	Pharmacia
DMSO	BDH
Dynabeads® Oligo $(dT)_{25}$	Dynal, Wirral, UK.
Expand™ High Fidelity/Long template PCR System	Boehringer-Mannheim
GF/C Glass Microfibre Filters	Whatman, Kent, UK.
Goat anti-rabbit Ig-peroxidase	Amersham, Buckinghamshire, UK.
Goat serum	Sigma
Hybond™- N^+ nylon membrane optimized for nucleic acid transfer	Amersham
Hydrogen Peroxide	Sigma

IPTG, X-gal	Alexis Corp. Ltd., Nottingham, UK.
Lambda DNA	NBL Gene Sciences Ltd., Northumberland, UK.
Lysozyme	Sigma
Microtitre 96 well plates	Falcon; Fahrenheit, Milton Keynes
Mineral oil	Sigma
Modifying enzymes	Gibco BRL, Renfrewshire, UK.
Nuc® Trap Probe Purification Columns	Stratagene, Cambridge, UK.
Oligonucleotide primers	Perkin Elmer Ltd., Cheshire, UK.
O.C.T compund (for microscopical cryotomy)	BDH Gurr®
PCR tubes (thin walled)	NUNC
Petri Dishes	Bibby Sterilin Ltd, Staffordshire, UK.
Phenol (tris-saturated)	Sigma
Phenol (water saturated)	Rathburn Chemicals Ltd., Walkerburn, UK.
Proteinase K	Boehringer-Mannheim Ltd.
Radioisotopes	Amersham
Random Primed DNA Labelling Kit	Boehringer-Mannheim
Rapid-hyb buffer	Amersham
Restriction endonucleases	NEB, Gibco BRL
RNA marker	Gibco BRL
RNase A	Boehringer-Mannheim
Salmon Sperm DNA	Gibco BRL
Sephadex™ G25 resin	Sigma
Sephaglas™ Band Prep Kit	Pharmacia Biotech
Sequagel® sequencing solutions	Flowgen Instruments Ltd.
Sequenase PCR Product Sequencing Kit	Amersham
Sequenase® Quick-Denature™ Plasmid sequencing kit	Amersham
Superscript™ reverse transcriptase	Gibco BRL
Sequencing tubes	Camlab, Cambridge, UK.
3,3′,5,5′-Tetramethyl benzidine	Sigma

T4 Polynucleotide kinase	NEB
T7 Quick Prime kit	Pharmacia Biotech
Tween®20	Boehringer-Mannheim Ltd.
Taq DNA polymerase	Promega, Southampton, UK.
Vectabond™ reagent	Vector Laboratories, Inc., Peterborough
VECTASHIELD® mounting medium	Vector Laboratories, Inc.,
Vent DNA Polymerase	NEB
Wizard™ Minipreps DNA Purification System	Promega
Wizard™ PCR Preps DNA Purification System	Promega
X-ray film	Genetic Research Instrumentation Ltd.,UK.

2.2 Bacterial Strains/Plasmid

STRAIN; (Supplier)	GENOTYPE	GROWTH MEDIA
E.coli TG1; (New England Biolabs Inc., Hertfordshire, UK)	K12 del *(lac,pro) supE, thi, hsdD5*/F′*traD36, pro AB*+, *lacI*^{q}, *lacZ* delM15	DYT
E.coli XL1 Blue; (Invitrogen, The Nederlands)	*supE 44, hsdR17, recA1, endA1, gyrA46, thi, relA1 lac* (F′ *ProAB*+, *LacI*^{q} , *LacZ*, delM15, Tn10 (tetR))	LB + 0.2% (w/v) maltose + 10 mM $MgSO_4$ + tetracycline (12.5 µg/ml)
SOLR; (Stratagene, Cambridge, UK)	*hsdR17, recB, recJ, uvrC, umuC,lac gyrA96,relA1, thi1, endA1*, λ^R (F′ *proAB, lacI*^{q}, *LacZ*, delM15) Su^-, Tn5 (kanR)	LB + kanamycin (50 µg/ml)

PLASMID; (Supplier)	GENOTYPE	GROWTH MEDIA
pBluescript™;(Stratagene)	ampR, 2.95 Kbp	LB + ampicillin (50 µg/ml)

2.3 Antibiotic solutions

ANTIBIOTIC	STOCK SOLUTIONS
Ampicillin	100mg ampicillin/ml dd water
Kanamycin	50mg kanamycin/ml dd water
Tetracycline	5mg Tetracycline Hydrochloride/ml 50% (v/v) absolute ethanol

Solutions were prepared in double distilled (dd) water or absolute ethanol, filter sterilised using a 0.22μm Millipore filter and stored at −20°C

2.4 Culture media

MEDIA	COMPONENTS (gm l^{-1})
Agar	1.5% (w/v) Bactoagar
DYT broth	16g tryptone, 10g yeast extract, 5g NaCl
LB-agar	1.5% (w/v) Bactoagar added to LB-broth. LB-agar plates: LB-agar was cooled to 40°C, mixed with the appropriate antibiotic, and poured into 90mm∅ sterile petri dishes.
LB-broth	10g tryptone, 5g yeast extract, 10g NaCl
NZY Agar	10g NZ amine (casein hydrolysate), 5g NaCl, 2g $MgSO_4$. $7H_2O$, 5g yeast extract, 15g bactoagar, (pH 7.5 with NaOH)
NZY Top Agar	10g NZ amine (casein hydrolysate), 5g NaCl, 2g $MgSO_4$. $7H_2O$, 5g yeast extract, (pH 7.5 with NaOH), 7g agarose

Bacterial culture media. All solutions were prepared in double distilled (dd) water and sterilised at 1.41 KPa for 20min.

2.5 Buffers, Dyes and Solutions

SOLUTION	COMPONENTS
Acrylamide	Commercially available solution of 30% (w/v) acrylamide/0.8% (w/v) bis-acrylamide; Stored at 4°C covered in foil
Artificial Perienteric Fluid (APF)	67mM NaCl; 67mM CH_3COONa; 3mM KCl; 3mM $CaCl_2$; 15.7mM $MgCl_2$ $6H_2O$, 5mM Tris; 3mM glucose, pH 7.6 with glacial acetic acid
Bromophenol blue loading dye	0.25% (w/v) bromophenol blue; 40% (w/v) sucrose; 1mM EDTA in sterile deionised water
Chloroform/iso-amyl alcohol	Chloroform/iso-amyl alcohol, 49:1 v/v; Stored at 4°C covered in foil
DEPC-water (RNase-free)	0.1% (v/v) DEPC added to dd water, mixed vigourously for 10min; left o/n at 37°C
Disodium hydrogen phosphate buffer	0.1M Na_2HPO_4, pH with 1M NaH_2PO_4
DNTP mix	10mM or 4mM dNTP mix prepared from 100mM stock solutions of dATP, dCTP, dGTP and dTTP; Stored at –20°C
Ethidium Bromide	10mg/ml ethidium bromide stock; Stored at 4°C covered in foil
Formaldehyde	Commercially available as a 37% solution (12.3M) in water;
Hydrogen Peroxide	Commercially available as a 30% (v/v) solution; Stored at 4°C
IPTG	100mM IPTG; Stored at –20°C
Paraformaldehyde (PFA)	4% (w/v) paraformaldehyde added to PBS in a screw capped bottle and heated to 60°C in a water bath. 2-4 drops of 1M NaOH added and solution heated until clear; pH adjusted to 7.0 with conc. HCl
PBS	140mM NaCl; 2.7mM KCl; 10mM Na_2HPO_4; 1.76mM KH_2PO_4, pH 7.4
Phenol/Chloroform Iso-amyl alcohol	25% (v/v) phenol equilibrated with 10mM Tris-HCl, pH 7.6; 24% (v/v) chloroform; 1% (v/v) iso-amyl alcohol; Stored at 4°C covered in foil
Phenol (Tris-saturated)	Commercially available or prepared as a solution equilibrated with 10mM Tris-HCl, pH 7.6; Stored at 4°C covered in foil
Potassium acetate	6M, (pH 5.0), 3M (pH 4.8) pot. acetate, pH adjusted with glacial acetic acid
SDS	20% (w/v), 10% (w/v) SDS
Sodium Acetate	3M, pH 5.2 or 2M, pH 4.0 sod. acetate; pH adjusted with glacial acetic acid
Sodium acetate – citrate buffer	1M Sodium acetate, pH 4.0 with 1M citric acid
Sodium azide	1% (v/v) sodium azide

Sodium hydrogen carbonate	0.5M $NaHCO_3$, pH 8.3 with 1M NaOH
TAE buffer	40mM Tris-acetate; 1mM EDTA, pH 8.0
Tris buffers	1M Tris base pH 7.5 to 9.0 adjusted with conc. HCl
10x TBE buffer	0.89mM Tris-HCl; 0.89M boric acid; 20mM EDTA, pH 8.0 with conc. HCl
TE buffer	10mM Tris-HCl; 1mM EDTA, pH 7.6 with conc. HCl
Tween®20	Commercially available as a 10% (v/v) solution; Stored at 4°C
X-gal	2% (w/v) X-gal dissolved in DMF (Sigma); Stored at 4°C covered in foil

All solutions were prepared in double distilled (dd) water, sterilised at 1.41 Kpa/20min and stored at room temperature unless stated otherwise

2.4 Primers

RoRidT	5′ GACTACGTTAGCATCTAGAATTCTCGAG$[T]_{17}$ 3′
M13 –20 primer*	5′ GTAAAACGACGGCCAGT 3′
T3 primer*	5′ AATTAACCCTCACTAAAGGG 3′

* Primers to match pBluescript

2.6 Nematode tissue supply

Ascaris suum worms were kindly provided by Dr. Richard J. Martin, Department of Preclinical Veterinary Sciences, University of Edinburgh, UK (See Section 2.7.2).

A.suum genomic DNA was kindly provided by Dr. Tim G. Geary, Pharmacia & Upjohn, Inc., U.S. Product Pharma Center, Research and Development, Kalamazoo, MI, U.S.A. (See Section 2.8)

Ivermectin susceptible and resistant *Haemonchus contortus* eggs and adult worms were provided by Dr. Gerald C Coles, Department of Clinical Veterinary Science, University of Bristol,UK. The eggs were collected from sheep faeces by the flotation method (Section 2.7.1).

The N2 strain of *Caenorhabditis elegans* was obtained from glycerol stocks of the same in the laboratory and grown (See Section 2.7.3) (Brenner, 1974).

(B) General Methods

2.7 Worms: Isolation/Maintenance/Growth

2.7.1A Flotation method of purification of *H. contortus* eggs

Uninfected sheep (crossbred Dorset) were experimentally infected with L3 *H. contortus* wild type isolates, susceptible to anthelminthics. The sheep were maintained in an indoor helminth-free environment. About 3 weeks post infection, sheep faeces were collected in polythene nappies. Faeces were homogenised in a blender with sufficient water to obtain a thin paste. This was washed through a 150μm sieve and the filtrate discarded. The liquid filtrate was centrifuged at 1500 x *g* in 50ml Falcon™ tubes for 2min and the pellet resuspended in saturated NaCl solution until a meniscus formed over the top of the tube. A large glass cover slip was placed over the top to give a tight seal. Tubes were then centrifuged again at 1500 x *g* for 2min. Cover slips with a film of eggs on them were removed quickly and washed off into a fresh Falcon™ tube. A sample was checked under the microscope. The eggs were used immediately or weighed into cryotubes and frozen under liquid nitrogen for further use

Ivermectin resistant *H. contortus* eggs were obtained from faeces of sheep dosed with ivermectin (0.2mg/Kg body weight) and infected with an IVM resistant isolate of the South African (White River) field isolate. A positive egg count (30 eggs/g or more) was used as a reference (Hong, Hunt and Coles, 1996). Eggs were isolated by the flotation method as described.

2.7.1B Isolation of adult *H. contortus* worms

Sheep infected with *H. contortus* were slaughtered in the abattoir, the intestines removed in the process, abomasum cut open, and semi digested contents removed by gently rinsing in water. The cleaned organ was placed in an isolation apparatus and live adults collected in PBS at 37°C. Live worms were placed immediately into fix or liquid nitrogen for use in immunocytochemistry or RNA extraction respectively.

2.7.2 Maintenance of adult *A. suum* worms

The worms were obtained from an abattoir where pig intestines were emptied for the preparation of sausage skins and placed in a flask containing artificial perienteric fluid, (APF) at 37°C. The worms were dissected out, placed in 1ml screw capped vials and stored under liquid nitrogen for further use. Live worms were placed in fix and used for immunocytochemistry.

2.7.3 Growth of *C. elegans* worms

REAGENTS	COMPONENTS (g/l)
NGM agar (Brenner, 1974)	3g NaCl, 2.5g peptone, 17g bacto-agar in one 975ml water. Cooled to 55°C and 25ml 1M KH_2PO_4, pH 6.0 and 1ml each of cholesterol (5mg/ml in ethanol), 1M $CaCl_2$, 1M $MgSO_4$ added aseptically
M9 buffer	6g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4$. $7H_2O$
1M potassium phosphate	136g KH_2PO_4, pH 6.0 with conc. KOH
Complete S basal medium	Filter sterilised solutions of 10ml of 1M potassium citrate, pH 6.0; 10ml trace metals solution; 3ml 1M $CaCl_2$; 3ml 1M $MgSO_4$ were added to one litre sterile S basal medium
S basal medium	5.9g NaCl; 50ml 1M potassium phosphate, pH 6.0; 1ml cholesterol (5mg/ml in ethanol); Autoclaved
1M potassium citrate	268.8g tripotassium citrate, 26.3g citric acid monohydrate, pH 6.0 with conc. KOH
Trace metals solution	1.86g Na_2EDTA, 0.69g $FeSO_4$. $7H_2O$, 0.20g $MnCl_2$. $4H_2O$, 0.29g $ZnSO_4$. $7H_2O$, 0.016g $CuSO_4$; Stored in dark

The N2 strain of *C.elegans* was routinely maintained on NGM agar plates seeded with OP50, a uracil requiring mutant of *E.coli*. Plates were incubated at 15°C and subcultured after every 10 days.

C.elegans worms were grown in large scale in liquid cultures on dense suspensions of *E.coli* OP50. An overnight culture of OP50 in DYT medium was spun at 3000 x *g* for 5min at room temperature and resuspended in complete S basal medium at a

concentration of 20g wet weight per litre. Two litre flasks with 500ml of the suspension were inoculated with 2 x 10^4 worms washed off plates, and cultures vigorously aerated at 20°C for about five days when they started to clear. The soup was transferred to a 1 litre glass cylinder and nematodes allowed to settle overnight at 4°C. The complete S basal medium was carefully drained and worms resuspended in cold 35% (w/v) sucrose and spun in a swinging bucket rotor at 700 x *g* for 5min in Falcon™ tubes. *C.elegans* worms that floated to the top were transferred to a fresh Falcon™ tube and washed twice with 0.1M NaCl. The yield was ~3-5g wet weight of nematodes per litre of culture.

2.8 Isolation of genomic DNA from worms

	REAGENTS	COMPONENTS
[A]Herman and Frischauf method	TEN9 buffer	50mM Tris-HCl, pH 9.0; 100mM EDTA; 200mM NaCl with 100µg/ml DNase-free RNase A
	Proteinase K	100mg/ml stock
[B] CTAB method	CTAB buffer	2% (w/v) CTAB; 0.1M Tris-HCl, pH 8.0; 2mM EDTA; 1.4M NaCl; 1% (w/v) polyvinyl pyrrolidone
	Precipitation buffer	1% (w/v) CTAB; 50mM Tris-HCl, pH 8.0; 10mM EDTA
[C] Improvised Proteinase K method	SET buffer	100mM NaCl; 20mM EDTA; 50mM Tris-HCl, pH 8.0

[A] Herman and Frischauf method

2g nematodes were ground to a powder under liquid nitrogen with a pestle and mortar and the powder quickly resuspended in 20ml of TEN9 buffer. The solution was transferred to a 50ml Falcon™ tube and shaken to give a homogenous suspension. 1ml each of 20% SDS (w/v) and proteinase K (10mg/ml) were added and mixed by inverting the tube. The tissue was digested o/n at 55°C and then transferred to a large, flat sided glass bottle (500ml). 20ml of 0.1M Tris equilibrated phenol (pH8.0), was added and the

bottle placed flat on a gently rocking platform (30rpm) for 1-3h. Phenol and aqueous phases were separated by centrifugation at 700 x *g* for 10min. The upper aqueous phase was drawn into a fresh bottle with a wide bore pipette. The phenol extraction was repeated until the aqueous phase was clear. Remaining debris was removed by centrifugation at 6300 x *g* for 20min at room temperature. The supernatant was dialysed against a 1000-fold volume of TE (pH7.6), first at room temperature, to avoid SDS precipitation, and then overnight at 4°C. DNA was precipitated by the addition of 0.1 volume of 3M sodium acetate, pH 5.5 and 0.8 volume of 2-propanol. The solution was mixed gently by inverting the tube several times. The DNA was spooled out with a glass rod, and placed into TE (1ml/gm tissue) in a microfuge tube. The tube was placed on a rotating wheel at 10rpm and DNA allowed to dissolve overnight at 4°C (Herman and Frischauf, 1987).

[B] CTAB method

This method has the advantage of removing unwanted carbohydrate compounds from genomic DNA preparations. CTAB acts as an efficient detergent and forms Schiff bases with amino acids, thus resulting in efficient separation from DNA (Vado *et. al.*, 1992). 2g nematode tissue was added to 10ml CTAB buffer at 65°C in a Falcon™ tube and incubated at 56°C for 15min in a shaking water bath until the tissue dissolved. 10ml of chloroform:iso-amyl alcohol (24:1) was added and the tube gently inverted to mix the contents. The tube was then spun at 10000 x *g* at 20°C for 10min. The aqueous phase was removed into a fresh Falcon™ tube and 0.2 volumes of 5% (w/v) CTAB added. The extraction was repeated. The clear aqueous phase was transferred to a 30ml Corex tube and an equal volume of precipitation buffer added. The tube was sealed, the contents gently mixed by inverting and spun at 700 x *g* at 20°C for 15min. The pellet was resuspended in 2ml TE buffer, pH 7.6; 1M NaCl and incubated at 56°C for 10min to enable it to dissolve. The tube was placed on ice and 2 volumes of pre-chilled 95% (v/v) ethanol added. The DNA was spooled out. Alternately, the tube was spun at 10000 x g at 4°C for 10min, pellet washed in 70% (v/v) ethanol and vacuum dried. The DNA pellet was finally suspended in 200µl of TE buffer, pH 7.6 at 4°C (Shahjahan *et. al.*, 1995; Steenkamp *et. al.*, 1994; Boyce *et. al.*, 1990).

[C] Improvised proteinase K method (Adlouni *et. al.*, 1995)

1gm *A. suum* tissue was powdered under liquid nitrogen and incubated in 10ml of SET buffer and 0.75ml of 20% (w/v) SDS for 60min at 65°C. 50µl of proteinase K (20mg/ml in SET buffer) was added and the sample incubated for 3h. This was followed by addition of 3.75ml of 6M potassium acetate. The sample was placed at 0°C for 30min to enhance protein precipitation and spun at 10, 000 x *g* for 10min at 4°C. An equal volume of 0.1M tris equilibrated phenol (pH 8.0) was added to the supernatant, the sample gently agitated for 10min and centrifuged for 15min at 4°C. The aqueous phase was transferred to a fresh tube and the extraction repeated with equal volumes of phenol-chloroform-isoamylalcohol (25:24:1, v/v/v) and chloroform-isoamyl alcohol (24:1, v/v). To the upper aqueous phase, two volumes of ice-cold ethanol (95%) was added and the mixture placed for 2h at –20°C. Precipitated DNA was spooled into a microfuge tube and spun at maximum speed for 15min at 4°C. The pellet was dried and dissolved in 0.5ml of TE buffer (pH 7.6). 5µl of RNaseA (20mg/ml) was added and the sample incubated at 37°C for 1h. Extractions using equal volumes of tris-buffered phenol, phenol-chloroform and chloroform alone were repeated. Two volumes of ice-cold ethanol was added to the aqueous phase resulting from the extraction process. The tube was inverted several times and left at –20°C overnight. The DNA sample was spun at 2000 x *g* for 15min at 4°C and the pellet washed with 70% (v/v) ethanol. The pellet was finally air dried and dissolved in required volume of TE buffer (pH 7.6).

2.9 RNA: Extraction/Detection

2.9.1 Isolation of total RNA

REAGENTS	COMPONENTS
Denaturing solution	4M guanidinium thiocyanate; 25mM sodium citrate, pH 7.0; 0.5% (w/v) sarcosyl; 0.1M 2-mercaptoethanol
Sarcosyl	1% (v/v) N-Lauryl sarcosine
SDS*	10% (v/v) SDS

* RNase-free solutions were prepared in 0.1% (v/v) DEPC treated water and sterilised at 1.41 KPa for 20min. SDS solution alone was prepared by addition of RNase-free SDS to sterilised DEPC treated water. Baked glassware was used for all purposes

Total RNA was isolated using the single step guanidinium thiocyanate-phenol chloroform extraction (Chomczynski and Saachi, 1987). Frozen tissue (1g) was ground to a powder under liquid nitrogen. The powder was rapidly dispersed in 10ml of denaturing solution and homogenised in a glass Teflon homogeniser. Sequentially, 1ml of 2M sodium acetate, (pH 4.0), 10ml of water saturated phenol, and 2ml of chloroform-isoamyl alcohol (49:1 v/v) were added, with thorough mixing after addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15min. The sample was centrifuged at 10,000 x *g* for 20min at 4°C. The aqueous phase was removed, mixed with 10ml of 2-propanol and left at –20°C for at least 2h. RNA was pelleted at 10,000 x *g* for 20min, and the pellet dissolved in 3ml of denaturing solution. RNA was again precipitated with 3ml of 2-propanol at –20°C for 1h. After centrifugation, the RNA pellet was suspended in 75% (v/v) ethanol, repelleted,0 and vacuum dried. Total RNA was finally suspended in 0.5ml of RNase free 0.5% (w/v) SDS and heated at 65°C for 10min.

2.9.2 Quantifying Recovered RNA

The amount of total RNA isolated was estimated by determining the $O.D_{260}$ of a 1:100 dilution of the sample in DEPC treated sterile dd water using a spectrophotometer. A 1:100 dilution of 0.5% (w/v) SDS in sterile dd water was used as a blank. The concentration was calculated using the equation:

RNA — Absorbance $_{260}$ x 40 x dilution factor

Standards: (1O.D unit for RNA corresponds to 40μg/ml)
(1O.D unit for DNA corresponds to 50μg/ml)

The $O.D_{280}$ was also determined to obtain a ratio for $O.D_{260}$/ $O.D_{280}$ absorbance as an indication of purity.

2.9.3 Analysis of RNA on Agarose gel

The integrity of the isolated total RNA was checked by non-denaturing agarose gel electrophoresis. A 1% (w/v) agarose gel of 60ml volume, with ethidium bromide (0.5μg/ml) was prepared in RNase-free 1X TBE buffer and run in a gel tank treated with

3% (v/v) hydrogen peroxide and washed with sterile dd water. A sample was prepared using 2-5µl each of the total RNA in 0.5% (w/v) SDS, RNase free BPB dye and DEPC-water. The gel was run in RNase-free 1x TBE buffer at a constant 120V and observed under ultraviolet illumination.

2.9.4 Purification of mRNA

REAGENTS	COMPONENTS
2x Binding buffer*	20mM Tris- HCl, pH 7.5; 1.0M LiCl; 2mM EDTA
Washing buffer*	10mM Tris-HCl, pH 7.5; 0.15M LiCl; 1mM EDTA
Elution buffer*	2mM EDTA, pH 8.0

* RNase-free solutions were prepared in 0.1% (v/v) DEPC treated water and sterilised at 1.41 KPa for 20min. Baked glassware was used for all purposes

Poly-$[A]^+$ RNA was selected using Dynabeads® oligo dT_{25} magnetic beads, 2.8µm in diameter, with a 25 nucleotide long chain of deoxythymidylate attached covalently to the bead surface via a 5' linker group. 0.2ml (1.0mg) bead suspension in a microfuge tube was placed into a Dynal MPC®-E magnetic particle concentrator (Uhlen, 1989). The supernatant was removed and the beads washed by adding 200µl of 2x binding buffer. The beads were again resuspended in 100µl of 2x binding buffer. Total RNA (~200µg) was dissolved in 100µl of DEPC-water, heated for 2min at 65°C, and added to the washed beads. The tube was left at room temperature for 5min to hybridise and then placed in the separator to remove the supernatant. The beads were washed twice with 200µl washing buffer and resuspended in 30µl of elution buffer before being heated at 65°C for 2min. Eluted poly $[A]^+$ RNA was extracted immediately after separation from the beads and used directly for cDNA synthesis. In case the eluted poly $[A]^+$ RNA required concentration to a smaller volume, $1/10^{th}$ and 21/2 volumes of RNase-free 3M sodium acetate (pH 5.4) and chilled absolute ethanol respectively were added to the tube and left overnight at –20°C. The tube was then spun in a microfuge for 30min at 4°C, the pellet dried in a Speed Vac for 3min and resuspended in required volume of DEPC water.

2.9.5 cDNA synthesis

REAGENTS	COMPONENTS
Superscript buffer*	50mM Tris-HCl, pH 8.3; 75mM KCl; 3mM $MgCl_2$
10x STE buffer	100mM Tris-HCl, pH 8.0; 10mM EDTA; 1M NaCl
10x alkaline electrophoresis buffer	0.3M NaOH freshly prepared, 20mM EDTA
2x alkaline electrophoresis loading dye	20% (v/v) glycerol; 4.6% (v/v) saturated bromophenol blue; 25mM NaOH

* RNase-free solutions were prepared in 0.1% (v/v) DEPC treated water and sterilised at 1.41 KPa for 20min. Baked glassware was used for all purposes

2.9.5[A] Reverse transcription

Poly-$[A]^+$ RNA was reverse transcribed into cDNA using Superscript™ reverse transcriptase enzyme, a recombinant reverse transcriptase from Moloney Murine Luekemia Virus (M-MLV) lacking RNaseH activity (Kotewicz *et al.*, 1988). To 25µl of eluted mRNA were added 10µl of superscript buffer (50mM Tris-HCl, (pH 8.3); 75mM KCl; 3mM $MgCl_2$), 5µl of 0.1M DTT, 2.5µl of 10mM dNTPs, 1µl RNasin (40U), 0.5µl of oligo RoRidT primer (See Table 2.4) and 1µl of Superscript™ reverse transcriptase (200U). The reaction was incubated at 37°C for 1h and stored at –20°C before further use (Levin and Simon, 1995). An aliquot of 4µl of the reverse transcription mix was used for radioactive monitoring of cDNA synthesis with [α-^{32}P] dATP.

2.9.5[B] Alkaline agarose gel- Sample preparation

cDNA synthesis was checked by preparing a sample with 2µl of 10x STE buffer, 4µl of the reverse transcription mix and 0.5µl of [α-^{32}P] dATP in a total volume of 20µl in an RNase-free tube. The reaction tube was placed at 37°C for 1h for incorporation of radiolabel. Unincorporated ^{32}P was removed by passing the sample through a Sephadex G25 column. The column was prepared by loading 1ml of Sephadex™ G25 matrix on to a 1ml syringe packed with siliconised glass wool in the bottom. The resin was added to give a bed volume of 1ml.

A λ *HindIII* marker was prepared on the side in a total volume of 20μl with 1μl of λ*HindIII* DNA (10μg/μl), 1μl of g [λ^{32}P]-ATP, T4-polynucleotide kinase (PNK) and 2μl of 10x T4-PNK buffer (350mM Tris-HCl, (pH 7.6); 50mM $MgCl_2$; 500mM KCl; 5mM β-mercaptoethanol) (Gibco). The reaction tube was left at 37°C for 1h. Unincorporated label was removed by passing the sample through a Sephadex G25 column as described earlier.

2.9.5C Alkaline agarose gel electrophoresis

0.8g of agarose was added to 72ml of dd water and heated. Upon cooling to 55°C, 8ml of 10x alkaline electrophoresis buffer was added, poured on a gel apparatus, and left to set. Samples were prepared in an equal volume of 2x alkaline electrophoresis loading dye. The agarose gel was run in 1x alkaline electrophoresis buffer at 100mA until the BPB dye migrated about three quarters distance of the gel from the well. The gel was then blotted by placing a stack of paper towels below 3mm Whatman filter paper, followed by the gel, a second stack of paper towels and a weight. The edge of the gel and filter were cut and the position of the wells on the membrane marked. The set up was left for 3h until water from the gel was blotted on to the towels. A Kodak X-ray film was placed on the gel covered in cling film and the autoradiogram exposed overnight.

2.9.6 Northern Blotting

REAGENTS	COMPONENTS
5x MOPS/EDTA buffer*	0.1M MOPS, pH 7.0; 40mM sodium acetate; 5mM EDTA, pH8.0 [20.6g MOPS dissolved in 800ml DEPC-treated 50mM sodium acetate, pH adjusted to 7.0 with 2N NaOH, 10ml DEPC-treated 0.5M EDTA (pH 8.0) added, total volume made up to 1litre. Stored in dark at room temperature; prepared fresh
Electrophoresis buffer*	1x MOPS/EDTA buffer
Sample buffer*	7.2ml deionised formamide, 3.2ml 5x MOPS buffer, 2.6ml deionised formaldehyde, 0.2ml water, 1ml 80% (v/v) glycerol, 0.8ml bromophenol blue dye (saturated solution in water); prepared fresh
Formaldehyde	Commercially available as a 37% solution (12.3M) in water
Staining solution	5μg/ml ethidium bromide in 1x MOPS buffer
Rapid-hyb buffer	Commercially available rate enhanced hybridization buffer for rapid hybridization of radiolabeled nucleic acid probes; 5x SSC, 5x Denhardt's solution, 0.5% (w/v) SDS
1x STE buffer	100mM NaCl; 20mM Tris HCl, pH7.5; 10mM EDTA

* RNase-free solutions were prepared in 0.1% DEPC treated water and sterilised at 1.41 KPa for 20min (except MOPS). Baked glassware was used for all purposes

2.9.6A Sample Preparation

Total RNA (Section 2.9.1) or mRNA isolated (Section 2.9.4) were transferred from an electrophoretically separated denaturing gel, on to a membrane, by Northern blotting (Meinkoth and Wahl, 1984). The sample was prepared by mixing 10μg total RNA or 1-2μg mRNA with 10-20μl of sample buffer containing BPB dye in an RNase-free tube. The tube was heated at 55°C for 15min immediately before loading. The RNA marker was prepared in the same way. All samples were prepared preferably in duplicates in order to allow for one set to be stained by ethidium bromide and the other to be blotted on to nylon membrane. An RNase-free DNA marker was also loaded for comparison.

2.9.6B Denaturing agarose gel electrophoresis

A 1% (w/v) agarose gel was prepared by adding 1g agarose to 20ml of 5x MOPS buffer and 75ml DEPC-water in an RNase-free conical flask. The agarose was dissolved and allowed to cool to 55°C. 5.4ml of formaldehyde (0.66M) was added and the gel mix poured into an RNase-free gel cast. The gel was allowed to set for 1h before use. Samples were loaded and the gel run in electrophoresis buffer in a tank treated with 3% (v/v) hydrogen peroxide and washed with sterile dd water at a constant 100V until the dye was two-thirds of the way down the gel. Two sets of RNA markers were loaded on the outside lanes of the gel.

2.9.6C Staining

At the end of the run, lanes containing one set of samples were cut from the gel and stained in ethidium bromide staining solution in a light proof container for 5min and destained in 500ml DEPC-water for 1h with three changes. The gel was then aligned next to a transparent ruler and photographed under ultraviolet illumination. The other half of the gel was not stained and used for blotting purposes.

2.9.6D Transfer

The gel to be blotted was washed in an excess of water for 15min and soaked in 10x SSC for 10min with two changes. All steps involved gentle shaking. A capillary blotting apparatus was setup to allow for RNA transfer overnight on to Hybond-N^+ membrane. A solid support covered with Whatman 3MM paper was allowed to dip in a reservoir with 10x SSC. The gel was placed inverted to allow quick and efficient transfer of DNA across the membrane followed by Clingfilm around the edges of the gel. Hybond-N^+ nylon membrane was placed on top of the gel, avoiding trapping air bubbles. Four pieces of Whatman paper were placed on top of the membrane, followed by a stack of paper towels, a flat surface plate and a 0.5 Kg weight. The blot was left for 4-12h. The orientation of the lanes on the gel were finally marked on the membrane which was air dried and baked for 2h at 80°C to fix the RNA on the membrane. The membrane was stored at 4°C before hybridization.

2.9.6E Hybridization, Probe Purification and Counting

The membrane was hybridized in required volume of preheated Rapid-hyb buffer (0.125ml buffer/cm^2 of membrane when blotted in bags) at 65°C for 30min with gentle agitation. Double stranded DNA probes were radiolabeled using the T7 Quick Prime kit. 25-50ng DNA suspended in a volume less than 34µl, was used with 10µl of Reagent mix provided in the kit (buffered aqueous solution containing dATP, dGTP, dTTP, and random oligo deoxyribonucleotides, primarily 9-mers), 185 x 10^{10} Bq (or 50µCi) of [α-^{32}P] dCTP. The final volume was made up to 49µl with distilled water. 1µl of T7 DNA Polymerase was added. The contents were spun down, and the tube incubated at 37°C for 15min.

Unincorporated nucleotides from the radiolobeled probe were removed by passing the sample through a Nuc Trap® probe purification column. A column containing resin was loaded with 70µl of 1x STE buffer. A syringe was fitted onto the column forming a seal between the two. The plunger of the syringe was then pushed home forcing the buffer down the column. The setup was dismantled and the labeled probe made up to a total volume of 70µl with STE buffer loaded on the resin at the top of the column. The syringe was carefully screwed onto the column and plunger pushed through it. The sample from the column was collected in a microfuge tube. 70µl of 1x STE buffer was loaded once again on the column and the eluant collected. The sample in a total volume of 140µl was used for subsequent steps.

5µl of the labeled probe was spotted onto the centre of a 2.5cmØ Glass Microfilter GF/C, the filter dried, and placed in a scintillation vial. ^{32}P counts were determined in the presence of 3ml toluene-based scintillation fluid and counting in the ^{32}P channel of a liquid scintillation analyser (1600 TR, Packard). This method of detecting ^{32}P was much more efficient than Cerenkov counting in the tritium channel which works only with a mean 25% efficiency (Maniatis, 1989).

The probe was denatured by boiling for 5min, snap cooled on ice and added to the Rapid-hyb buffer.

2.9.6F Washes

The hybridized membrane was washed in a solution of 1x SSC; 0.1% (w/v) SDS at 65°C for 15min, using 2-5ml/cm^2 of membrane. A second wash was done at 65°C in a solution of 0.1x SSC, 0.1% (w/v) SDS for 15min. The membrane was then exposed to a Kodak X-Ray film preflashed with a Mecablitz 45 C11 Metz Preflash (working aperture 5.6, operating range 0.5m) and placed at −70°C in a film cassette with screens. The autoradiogram was developed after a 24h exposure.

2.10 DNA: Isolation/Purification

2.10.1 Plasmid DNA preparation

	REAGENTS	COMPONENTS
[A] Alkali lysis method	GTE buffer	50mM glucose; 25mM Tris-HCl, pH 8.0; 10mM EDTA
	Detergent solution	0.2M NaOH; 1% (w/v) SDS
	Neutralisation solution	600ml 5M potassium acetate, 115ml glacial acetic acid; (3M with respect to potassium and 5M with respect to acetate)
[B] Wizard DNA™	Resuspension buffer	50mM Tris-HCl, pH 7.5; 10mM EDTA; 100µg/ml RNaseA
	Lysis solution	0.2M NaOH; 1% (w/v) SDS
	Neutralisation solution	2.55M potassium acetate; pH 4.8
	Column wash solution	200mM NaCl; 20mM Tris-HCl, pH 7.5; 5mM EDTA; 50% (v/v) ethanol
[C] Rapid Boiling Method	STET buffer	8% (v/v) sucrose; 5% (v/v) Triton X-100; 5% (v/v) glycerol; 50mM Tris-HCl, pH 8.0; 50mM EDTA

[A] Plasmid preparation (Alkali lysis method)

The alkaline lysis method (Sambrook, 1989; Birnboim and Doly, 1979) was used to isolate plasmid DNA from 1.5ml of overnight cultures in the appropriate antibiotic.

Cultures were spun at 13000 x *g* for 5min and pellet resuspended in 100µl of GTE buffer followed by addition of 200µl of detergent solution, with gentle inversion of the tube each time to mix the contents. The tubes were left on ice for 5min, 150µl of chilled neutralisation solution added and tubes vortexed. After another 5min on ice, tubes were spun at 13000 x *g* for 5min. The supernatant was transferred to a fresh tube, an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) added, the tube vortexed for 2min and spun at 13000 x *g* for 5min. The clear supernatant was removed into a fresh tube and DNA precipitated by the addition of two volumes of absolute ethanol at –20°C. The tubes were spun for 10min, DNA pellets washed with 500µl of 70% (v/v) ethanol and dried under vacuum. The pellet was finally resuspended in 50µl of TE buffer (pH 7.6) with 10µg/ml RNase A.

[B] Wizard DNA™ Miniprep

3ml of an overnight bacterial culture grown at 37°C in the appropriate antibiotic was centrifuged and used with the Wizard™ minipreps DNA purification kit. The pellet was suspended in 200µl resuspension buffer followed by 200µl of lysis buffer and neutralisation solution, with mixing several times after each addition. The clear lysate was collected after spinning the tube at 13,000 x *g* for 5min. 1ml of Wizard™ DNA purification resin was added to the supernatant and mixed in a 2ml syringe barrel attached to a Wizard™ minicolumn. The resin-lysate mix was gently pushed through the column with the syringe plunger. The minicolumn was then detached, the plunger withdrawn and the syringe barrel reattached to add 2ml of column wash solution. The mini-column was once again detached and spun at 13,000 x *g* for 2min to dry the resin. Plasmid DNA was eluted into a fresh tube by adding 50µl of water and spinning at 13,000 x *g* for 20sec. DNA fragments >3Kbp were eluted with water at 65°C-80°C. An aliquot was run on a 1% (w/v) agarose gel in TBE. The yield of DNA was typically 0.4µg/µl.

[C] Rapid isolation of plasmid DNA by boiling

The boiling method was used as a simple and rapid technique for isolating bacterial plasmids (Holmes and Quigley, 1981). 1.5ml of an overnight culture grown at 37°C in the

appropriate antibiotic was pelleted at 300 x *g* for 5min and resuspended in STET buffer, followed by addition of 25µl of freshly prepared lysozyme (10mg/ml). The suspension was heated for 40sec (not longer) in a boiling water bath and tube centrifuged at 12000 x *g* for 10min at room temperature. The supernatant was resuspended in equal volume of iso-propanol and DNA precipitated at –20°C for 10min. The tube was spun at 12000 x *g* for 5min at 4°C, the pellet washed in 500µl of 70% (v/v) ethanol, air dried and finally suspended in 50µl of TE buffer. 10µl was run on an agarose gel. The yield of DNA was typically 0.3µg/µl.

2.10.2 DNA purification

	REAGENTS	COMPONENTS
[A] Agarose gel electrophoresis	BPB loading Dye	15% (v/v) Ficoll, 0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol
	Marker	200µl Lambda DNA (0.3µg/ml) digested with 10µl enzyme λ*PstI* (NEB), 24µl Buffer 3 (50mM Tris-HCl; 10mM $MgCl_2$; 100mM DTT; pH 7.9) in total volume 240µl at 37°C; Reaction stopped with 10µl 0.1M EDTA; Stored at 4°C
[B] Sephaglas™ Band Prep kit	Modified gel solubilizer	Buffered solution containing NaI with 5µl of 50% (v/v) glacial acetic acid
	Wash buffer	20mM Tris-HCl, pH 8.0; 1mM EDTA; 0.1mM NaCl ; 18ml absolute ethanol added before use
	Elution buffer	10mM Tris-HCl, pH 8.0; 1mM EDTA
[C] Wizard™ purification of PCR products	Direct purification buffer	50mM KCl; 10mM Tris-HCl, pH 8.8; 1.5mM $MgCl_2$; 0.1% (v/v) Triton X-100

[A] Agarose gel electrophoresis

1-3% (w/v) non-denaturing agarose gels in 1x TBE buffer with 10µl ethidium bromide (10mg/ml) were run with 20µl of the DNA sample in 5µl BPB loading dye. λ/*Pst* was used as a size marker. Gels were typically run at a constant 120V for 1h prior to visualisation under UV illumination

[B] Sephaglas™ purification of DNA

The band of interest was cut from the gel using a razor blade. The Sephaglas™ Band Prep kit was used to purify DNA from the gel. 250µl of modified gel solubilizer was added to 0.25g of agarose (or 1µl for each mg of agarose) containing the band of interest. The tube was incubated at 60°C for 10min to dissolve the agarose. 5µl of Sephaglas BP suspension was added per µg of DNA and the tube incubated at room temperature for 5min, with periodic mixing, followed by a 30sec pulse spin in a microfuge. The pellet was resuspended in 40µl of wash buffer. After three washes in all, the pellet was air dried for 10min, resuspended in 20µl elution buffer and incubated for 5min at room temperature. The tube was centrifuged for 1min, and supernatant containing eluted DNA, collected into a fresh tube.

[C] Rapid purification of DNA fragments

The Wizard™ PCR Preps System was used to purify double stranded PCR amplified DNA from a high melting agarose gel when non-specific amplification products were to be removed, or directly from the PCR reaction when only a single specific product was noted. To 100µl of direct purification buffer in a 1.5ml eppendorf tube, 30-300µl of the aqueous phase of the PCR reaction separated from mineral oil was added. The tube was vortexed briefly and 1ml DNA purification resin™ added and the tube vortexed again, three times over a one min period. The resin/DNA mix was pipetted into a 2ml syringe barrel attached to a Wizard™ minicolumn and gently pushed through the column with the syringe plunger. The minicolumn was then detached, the plunger withdrawn, and the syringe barrel reattached to add 2ml of 80% (v/v) iso-propanol. The mini-column was once again detached and spun at 12,000 x *g* for 20sec to dry the resin. DNA was eluted into a fresh tube by addition of 50µl of water to the column and waiting for 1min and spinning at 13,000 x *g* for 20sec. DNA fragments >3Kbp were eluted with water at 65°C-80°C. An aliquot was run on a 1% (w/v) agarose gel in TBE. A 95% recovery was expected for fragments >500bp in size.

2.10.3 Phenol extraction and ethanol precipitation of DNA

DNA was purified by phenol extraction using equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1). The tube was vortexed and centrifuged at 10000 x g for 2min. The upper aqueous layer was transferred to a fresh tube and the DNA precipitated by the addition of 0.1 volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of chilled absolute ethanol. The tubes were left 20°C for 1h or longer and DNA pelleted by centrifugation at 15,000 x *g* for 20min at 4°C. The DNA pellet was washed with 500µl of chilled 75% (v/v) ethanol and air dried in a Speed Vac Concentrator (Model SVC-100H) for 5min. The pellet was finally suspended in 10µl of TE buffer (pH 7.6).

2.10.4 Restriction Digests

Restriction digests of DNA (1-5µg) were typically carried out in presence of 2µl of 10x restriction enzyme buffer and 1µl of the enzyme (typically 10U/µl) in a total volume of 20µl. Double digests were carried out in a buffer resulting in maximum activity of both enzymes. BSA was added when required. The mixture was incubated at 37°C for 1-12h.

2.11 Polymerase Chain Reaction

2.11.1 Primer design

Primers were in general designed with the following common parameters (Dieffenbach *et al.*, 1993): 1) The length of the primer was a minimum of 18 nucleotides. Each additional nucleotide made the primer four times more specific. 2) The 3′ end of the primer was critical and had a minimal mismatch in the last 5 to 6 nucleotides. 3) The terminal nucleotide in the primer had a G or C residue. 4) Primer pairs particularly at 3′ ends were not complementary to each other in order to avoid formation of primer-dimers. 5) Primers containing linker-adaptors on the 5′ end contained restriction enzyme sites that did not cut elsewhere within the DNA. 6) Primers were best designed with ~ 50% GC content. Primer pairs were designed such that their GC content and Tm (melting temperature, defined as the dissociation of the primer/template duplex) were close. 7) A

general and rough approximation calculation of the Tm was done by using the equation (Suggs *et al.*, 1981)

$$Tm = 4\,(G+C) + 2\,(A+T) \qquad (1)$$

The Tm was also calculated by using the equation (Lowe *et al.*, 1990)

$$Tm = 81.5+16.6\,(\log 10\,(Na^{+})) + \frac{41\,(G+C) - 675}{A+G+C+T}$$

where Na^{+} conc. is assumed to be 0.1M.

Although equation (2) is recommended for calculating the Tm of primers longer than 18bp, it was observed that PCR amplifications using Tm calculated from equation (1) as a reference gave better results.

2.11.2 Oligonucleotide synthesis and deprotection

Oligonucleotide primers for PCR were synthesised by the phosphoramidite principle method using the Applied Biosystems 381A DNA synthesiser. Reactive nucleosides reacted with the 5'-hydroxy group on a derivatised solid support. The activated phosphoramidite derivative was oxidised and resulted in an oligonucleotide bound to the support. Manual deprotection of exocyclic amines of the bases of the synthesised oligonucleotide was carried out to result in active DNA. The cartridge containing the solid was emptied into a screw capped tube and 1ml of conc. ammonium hydroxide solution added. The tube was left o/n at 55°C. The sample was transferred to a 15ml Corex® tube on ice and 1ml of glacial acetic acid added slowly, followed by 6ml absolute ethanol. The tube was centrifuged at 10000 x g for 5min, pellet washed with 1ml of 85% (v/v) ethanol, air dried and dissolved in 1ml of dd water. $O.D_{260}$ was measured to estimate the yield. Primer concentration was estimated using the equation:

$$\text{Primer conc. } (\mu M) = \text{Dilution factor} = \frac{O.D_{260}}{0.01 \times \text{length of primer in nucleotide residues}}$$

Primers were also obtained from Perkin-Elmer's Applied Biosystems Division. The custom synthesised primers were provided in acetonitrile and water ready for use.

2.11.3 Polymerase Chain Reaction (PCR)

	Taq DNA polymerase	**Expand™ High Fidelity PCR system**	**Vent $_R$® DNA polymerase**
Enzyme Source	*Thermus aqaticus* Strain YT1 (Chien *et al.*, 1976)	Enzyme mixture containing *Taq* and *Pwo* polymerases (Barnes, 1994)	*Thermococcus litoralis* (Perler *et al.*, 1992)
Enzyme added	0.5µl (5000U/ml)	0.75µl (3500U/ml)	0.5µl(2000U/ml)
Reaction Buffer	1x Reaction buffer - 100mM Tris-HCl, pH 9.0 @25°C; 500mM KCl; 1% Triton® X-100	10x High Fidelity buffer	1x Thermo Pol buffer -
DNTP	0.2mM	0.2mM	0.2mM
Primer conc. (downstream and upstream)	1µM	0.3µM	1µM
Optimal Mg^{2+} conc.	1.5mM	1.5mM	2mM

PCR was performed on a Perkin Elmer Cetus DNA-Thermocycler or on a PTC-100™ (Programmable Thermal controller); MJ Research, Inc., using thin-walled 0.5ml PCR tubes. PCR reactions were carried out using the thermostable enzyme *Taq* DNA polymerase, Vent$_R$® DNA polymerase or the Expand™ High Fidelity PCR System. *Taq* DNA polymerase frequently introduces a single deoxyadenosine residue that is template independent at the 3′ end of the PCR fragment via its terminal transferase activity. This allows for direct cloning into a plasmid T-vectors system (Marchuk *et al.* 1991). The Vent $_R$® DNA polymerase and Expand High fidelity PCR System have an advantage in that they possess higher fidelity than noted for *Taq* DNA polymerase through an improved 3′→5′ exonuclease proof reading activity. Fragments of sizes up to 5Kbp can be amplified with ease.

The concentrations of the individual components varied with the type of enzyme used, as listed in the above Table. The PCR reaction mix was finally overlaid with 50µl

mineral oil and subject to PCR cycling conditions. A typical example of a reaction set up consisted of

1µl cDNA or genomic DNA (≤ 10ng) template
10µl Reaction buffer (10x)
3µl $MgCl_2$ (25mM stock)
5µl 4x dNTPs (4mM stock)
5µl sense primer (20µM stock)
5µl antisense primer (20µM stock)

made up to a total volume of 100µl with dd water. No template and single primer controls were run on the side. PCR conditions included a hot start of 95°C for 5min (Dorfman, 1993) followed by addition of enzyme through the oil. The reaction tube was then subject to 40 cycles of denaturation at 95°C for 1min, annealing at 60°C for 1.5min, and extension at 72°C for 2min. A final extension of 72°C for 10min was carried out. Conditions varied in different PCR reactions were concentrations of DNA, of magnesium from 1-4mM, PCR cycling times and annealing temperatures depending on the Tm of the two primers used.

2.11.4 Touchdown PCR

This method of PCR allows for greater specificity and with amplification of desired targets in an exponential manner (Don *et al.*, 1991). The reaction set up was similar to a standard PCR reaction (Section 2.11.2) except that the annealing temperature initially began at a slightly higher temperature compared to the Tm, and was incrementally lowered for example by 0.5°C/cycle to an annealing temperature at which non-specific amplification occurred. The initial incremental lowering allowed for preferential priming of the primer-template combination, making it amplification specific (Roux, 1994). A typical example of touchdown-PCR conditions were

	Hot Start	95°C	4min
1.	Denature	95°C	30sec
2.	Annealing	60°C	50sec; falling by 0.5°C/cycle
3.	Extension	72°C	1.5min
	20 cycles of Steps 1 to 3.		
4.	Denature	95°C	30sec
5.	Annealing	50°C	50sec
6.	Extension	72°C	1.5min
	30 cycles of Steps 4 to 6		
	Final extension	72°C	7min

2.11.5 Inverse PCR

Inverse PCR was carried out on circularised genomic DNA or cDNA to amplify 5'ends of cDNA sequences (Ochman *et. al.*, 1988; Triglia *et. al.*, 1988). A sense primer was made to the 3'end of the cDNA sequence and an antisense primer to the furthermost 5' sequence available. PCR amplifications were performed as described in Section 2.10.2.

To circularise genomic DNA, 20µg was digested with a restriction enzyme such as *Eco RI* at 37°C overnight. DNA was ethanol precipitated (See Section 2.9.3) and the pellet resuspended in 792µl of dd water. 200µl of 5x T4 DNA ligase buffer (250mM Tris-HCl, pH 7.6; 50mM $MgCl_2$; 5mM ATP; 5mM DTT; 25% (w/v) PEG-8000) (Gibco BRL) and 8µl of T4 DNA ligase (5U/µl) were added, the contents mixed and the tube incubated at 16°C for 50h. The tube was spun at 10000 x *g* for 1h and DNA once again ethanol precipitated. The pellet was vacuum dried and suspended in 50µl of water. This was used for inverse PCR.

2.12 Cloning DNA/PCR products

2.12.1 Preparation of competent cells

Competent cells were prepared by the calcium chloride method (REF). 50ml of DYT medium was inoculated with 0.5ml of a fresh overnight culture of TG1 or XL1 cells

in a 250ml flask, and shaken at 37°C to an $O.D^{550}$ of 0.3.(~1.5h). Upon reaching the required O.D., the flask was chilled on ice and then transferred into 30ml Corex® centrifuge tubes. Cells were spun at 700 x *g* in a Sorvall® RC-5B centrifuge, SS-34 rotor for 10min at 4°C and resuspended in 20ml of chilled 50mM $CaCl_2$ at 4°C. After 20 min on ice, the cells were spun at 700 x *g* for 5min at 4°C, and the pellet resuspended in 3ml of ice cold 50mM $CaCl_2$. Competent cells were aliquoted into 300µl volumes in prechilled tubes and used for transformation. Competent TG1 cells were occasionally stored overnight while XL-1 cell were used immediately.

2.12.2 Transformation

5µl of the transforming DNA (~50ng) was added to 300µl competent cells. The tubes were placed on ice for 40min. Cells were heat shocked at 42°C for 1.5min and chilled on ice for 5min. 0.7ml of DYT was added and tubes placed in a shaker at 37°C for 1h. $1/10^{th}$ volume of the sample was plated directly. The remaining $9/10^{th}$ volume of the sample was spun at maximum speed in a microfuge for 5min, the pellet resuspended in 100µl DYT and plated on LB plates with 100µg/ml ampicillin. For blue/white selection of colonies, 50µl of 2% (w/v) X-gal in DMF and 10µl of 100mM IPTG were spread on LB-ampicillin (100µg/ml) plates and then cells plated. The plates were incubated inverted for 14-16h at 37°C and then examined. Bacterial cells producing a functional β-galactosidase protein due to the presence of an open reading frame encoding the LacZ alpha peptide, resulted in blue colonies in the presence of the chromogenic substrate X-Gal (Horowitz *et al.* 1964). Cells transformed with vector containing the insert resulted in white colonies due to disruption of the alpha peptide open reading frame. White colonies were therefore picked streaked on a fresh LB/amp (100µg/ml) plate and grown at 37°C.

2.12.3 Blunt-ended subcloning of DNA/PCR products

PCR products were phosphorylated (as they lacked a 3′ phosphate group) before blunt-ended cloning into an appropriate vector. 1.5µl of DNA polymerase I (5U/µl) (NEB) and 1µl of T4 Polynucleotide Kinase (10U/µl) (NEB) were added to a reaction

tube containing 15µl of gel purified insert DNA (10ng-1µg depending on the insert:vector ratio required), 2µl of 10x One-Phor-All Buffer Plus (Pharmacia) (100mM Tris-acetate, pH 7.5; 100mM magnesium acetate; 500mM potassium acetate) and 1µl of ATP (10mM). The contents were mixed and tube incubated at room temperature for 3min. 1µl of 4x dNTP (4mM) was then added and the mix incubated at 37°C for 30min. The reaction was finally stopped with 2µl of 0.5M EDTA (pH 8.0). The total volume was made up to 100µl with sterile dd water and DNA ethanol precipitated (See Section 2.10.3).

10µg of pBluescript SK^+ vector was digested with 3µl of *SmaI* (10U/µl), 1/10th the volume of 10x buffer A (10mM $MgCl_2$; 1mM DTT; 10mM Tris-HCl, pH 7.4; 50mM NaCl) (Boehringer) in a 50µl volume at 30°C for 1h. *SmaI* was heat inactivated at 75°C for 20min and the tube allowed to cool to room temperature for 5min. *Sma* cut vector was then dephosphorylated with 10µl of calf intestinal phosphatase (1U/µl) and 10x dephosphorylation buffer (25mM Tris-HCl, pH 7.6; 1mM $MgCl_2$; 0.1mM $ZnCl_2$; 50% (v/v) glycerol) at 37°C for 20min. The phosphatase was inactivated by addition of 5µl of 0.5M EGTA (pH 8.0) and heating to 65°C for 10min. Vector DNA was also extracted with phenol-chloroform and then ethanol precipitated. Both insert and vector DNA were run on a 1% non-denaturing agarose gel and the quantity estimated (See Section 2.10.2) before ligation.

2.12.4 Ligation

~200ng of purified linearised vector DNA was mixed with insert PCR product in a total volume of 20µl. Reactions were set up with molar insert:vector ratios from 1:1 to 5:1. The mixture contained 4µl of 5x T4 DNA ligase buffer (250mM Tris-HCl, pH 7.6; 50mM $MgCl_2$; 5mM DTT; 5mM ATP, 25% (w/v) PEG-8000) and 1µl of T4 DNA ligase (1U/µl), (Gibco, BRL). The ligation mix was incubated at room temperature for 12h. 5µl of the ligation mix was used to transform competent *E. coli* cells (Section 2.12.1-2). Control reactions were set up in parallel with (1) 1µl of the processed vector alone in the presence of T4 DNA ligase and ligase buffer and (2) 1µl of the processed vector alone in presence of ligase buffer in a total volume of 20µl. The reaction tubes were incubated overnight at 16°C.

2.12.5 Sticky ended cloning

Sticky ended ligations were carried out with vector and insert DNA that were double digested (Section 2.10.4), phenol-chloroform extracted and ethanol precipitated (Section 2.10.3) and ligated as described above.

2.13 DNA Detection

2.13.1A Southern blotting

REAGENTS	COMPONENTS
Denaturing Solution	1.5M NaCl; 0.5M NaOH
Neutralising solution	1.5M NaCl; 0.5M Tris-HCl, pH 7.2; 0.001M Na_2EDTA
100x Denhardt's	2% (w/v) ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) bovine serum albumin; Stored at –20°C
Prehybridization solution	5x SSC; 5x Denhardt's solution; 0.5% (v/v) SDS; 20µg/ml heat denatured salmon sperm DNA
20x SSC	3M NaCl; 0.3M $(CH_3COO)Na_3$, pH 7.0 with conc. HCl

DNA fragments, separated by agarose gel electrophoresis were transferred on to a Hybond™-N^+, positively charged Nylon membrane by the method of Southern (1975). After electrophoresis, the gel was soaked in two volumes of denaturing solution for 30min, with gentle shaking at room temperature in order to denature the DNA. The gel was then neutralised in neutralising solution for 30min with two changes, with shaking at room temperature. A capillary blot was set up and DNA transferred on to the membrane as described in Section 2.8.6D

2.13.1B Oligonucleotide labeling

The membrane was hybridized in a Perspex box for 8h in 50ml of prehybridization solution, with gentle shaking. DNA probes were labeled and unincorporated label removed as described in (Section 2.9.6E) and used for hybridization at 65°C. Synthetic oligonucleotide probes were labeled in a reaction mix of 2µl of 10x T4 polynucleotide kinase buffer (10U/µl) (700mM Tris-HCl, pH 7.6; 100mM $MgCl_2$; 50mM DTT), 2µl of

10μM oligonucleotide primer, 50μCi of [γ-^{32}P] ATP and 1μl of T4 polynucleotide kinase (NEB) in a total volume of 20μl. The tube was incubated at 37°C for 1h. The labeled oligonucleotide probe was added to the membrane and hybridized at a temperature 5°C below the Tm of the primer (Mason and Williams., 1988).

2.13.1C Washes

The hybridized membrane was washed three times in 50ml of 6x SSC; 0.1% (w/v) SDS, for 20min each at the hybridization temperature, and then for 2min at the Tm when a synthetic oligonucleotide was used as a probe. In the final wash, the blot was monitored with a Geiger counter each min and removed when the background level dropped below 25cps.

When a double stranded DNA probe was used, the hybridized membrane was washed in 50ml of 2x SSC, 0.1% (w/v) SDS for 30min at room temperature with three changes, followed by 50ml of 2x SSC, 0.1% (w/v) SDS for 30min at 65°C. Membranes were autoradiographed as described in Section 2.9.6F.

2.13.2 Ultrasensitive detection of nucleic acids by silver stained gel

REAGENTS	COMPONENTS
7.5% gel	2ml 5x Loening 'E' buffer, 2.5ml bis-acrylamide (0.8%/30% v/v), 100μl 10% (w/v) APS (fresh), 20μl TEMED made up to a total volume of 10.12ml
5x Loening 'E' buffer	180mM Tris-HCl, pH 7.8; 193mM NaH_2PO_4; 5mM EDTA
Running buffer	1x Loening 'E' buffer
Fix solution	10% (v/v) ethanol, 0.5% (v/v) acetic acid
Silver nitrate solution	17.6mM silver nitrate; (prepared fresh)
Developer	0.75M NaOH, 0.75% (v/v) formaldehyde; (prepared fresh)
Stop solution	0.09M $NaHCO_3$

A polyacrylamide gel was run to obtain an estimate of the concentration of cDNA/PCR products when obtained in low amounts. This made use of the fact that silver ions form a stable complex with nucleic acids (Jenson and Davidson, 1966), and detected nanograms of nucleic acid (Somerville and Wang, 1981,) apart from the ultrasensitive detection of polypeptides (Oakley *et al.*, 1980). Samples were prepared in 3µl BPB loading and a total volume of 10µl and loaded on a 1mm thick 7.5% SDS-PAGE gel poured in a 10 x 12cm 'Atto' gel rig, assembled with ethanol polished glass plates. Samples were electrophoressed through the gel using the Atto™ Mini-gel system in running buffer at a current of 20-40mA for 11/2h till the BPB dye was at the bottom of the gel.

Silver staining of the gel was carried out as described by Herring *et al.*, 1982. The gel was washed in 50ml fix solution for 15min and stained in 100ml freshly prepared silver nitrate solution for 15min with gentle shaking. The gel was then rinsed briefly in dd water, and this drained to add the developer. The gel was constantly shaken for a maximum of 10min until the bands were clearly visible as a result of reduction of silver ions, after which it was drained to add the stop solution. This step increased the intensity of staining of both the bands and the background. The stop solution was poured off after 5min and the gel photographed under transmitted light. For long term storage, the gel was placed in fix solution and dried.

2.13.3 DNA sequencing

2.13.3A Sample Preparation

REAGENTS	COMPONENTS
Enzyme dilution buffer	10mM Tris-HCl, pH 7.5; 5mM DTT; 0.5mg/ml BSA
Labeling mix (dGTP), 5x concentrate	7.5µM dGTP, 7.5 µM dCTP, 7.5µM dTTP
5x sequencing reaction buffer	200mM Tris-HCl, pH 7.5; 100mM $MgCl_2$; 250mM NaCl
Stop solution	95% (v/v) formamide; 20mM EDTA; 0.05% (v/v) BPB; 0.05% (v/v) xylene cyanol FF

DNA sequencing was carried out using Sequenase version 2.0 T7 DNA polymerase (Tabor and Richardson, 1987) based on the chain-termination method (Sanger *et al.*, 1977). 20µl (0.2µg/µl) of double stranded template DNA was denatured in 5µl of

freshly prepared 1M NaOH at room temperature for 5min. The sample was loaded on a column packed with Sepharose CL6B resin. The column was prepared by piercing a hole in the bottom of a 0.5ml microfuge tube with a syringe. 200µl of baked glass beads (Glasperlin 0.45 x 0.50 mm) were added followed by 1ml of CL6B resin. The eluant was allowed to drip into a larger 1.5ml eppendorf tube. The column was spun in a swinging rotor for 3min at 300 x *g*. This was repeated with the addition of 1ml of dd water. The sample was finally loaded and the eluant collected in a fresh tube. An aliquot of the eluant was checked for neutral pH on pH paper and then used as template for the annealing mixture in the sequencing reaction.

The annealing mixture was prepared with denatured DNA template in 7µl volume, 2µl of 5x sequencing reaction buffer and 1µl of primer (2pmol.). M13 reverse primer and KS primer designed to match the sequence in the multiple cloning site in pBluescript SK^+ vector were used to sequence inserts cloned therein. The reaction tube was placed at 65°C for 2min and cooled slowly to 35°C over 15-30min. The tube was spun briefly and chilled on ice until further use in the labeling reaction. In addition, four tubes with 2.5µl of termination mixture, each containing a single di-deoxy NTP as one of its components, labeled G,A,T,C were prewarmed in a 37°C water bath.

The labeling reaction was prepared by adding

1µl	DTT (0.1M)	
2µl	diluted labeling mix;	(diluted 1:5 in water)
0.5µl	[^{35}S]dATP (5µCi)	
2µl	diluted Sequenase Version 2.0 enzyme;	(diluted 1:8 in ice-cold enzyme dilution buffer)
10µl	ice cold annealed DNA mixture	

and the reaction tube incubated at room temperature for 3min.

The reaction was terminated by adding 3.5µl of the above mixture to each of the termination tubes, mixing the contents, and continuing incubation of the reactions at 37°C for 5min. 4µl of stop solution was finally added. Samples were heated to 75°C for 2min immediately before loading onto a sequencing gel.

2.13.3B Sequencing Urea-Polyacrylamide gel

REAGENTS	COMPONENTS
Binding mixture	1.2ml of 10% (v/v) glacial acetic acid, 200µl Silane A-174 adhesion promoter, 25ml absolute ethanol
6% Urea-PAGE gel	12ml concentrate, 33ml diluent, 5ml buffer (Sequagel reagents); 30µl TEMED; 300µl 16% (w/v) APS solution (prepared fresh)
Wash mixture	10% (v/v) glacial acetic acid; 10% (v/v) methanol
20x glycerol tolerant buffer	1.8M Tris base; 0.6M taurine; 0.01M Na_2EDTA

A 6% urea-polyacrylamide gel (PAGE) was prepared for running the sequencing reactions. Sequencing plates (40 x 20 cm) which were soaked overnight in 5M KOH, were washed thoroughly in water and polished with absolute ethanol. The horizontal backplate was treated with the binding mixture for 5min while the front plate was treated with dichlorodimethyl silane (BDH). Both plates were rinsed in water and ethanol polished. The two sequencing plates were then glued or clamped with a 0.2mm spacer in between them. A 6% urea-PAGE gel was prepared from Sequagel reagents and poured at once between the glass plates. The gel was left to set for 30min with the teeth edge of a 0.4mm thick comb inserted to form wells. After the gel was set, the comb was removed and wells washed thoroughly with 1x TBE buffer to remove any residue. Sample G, A, T, C tubes were denatured at 70°C for 2min and loaded on to each lane in that order on a Anachem Origo DNA Sequencing Unit. The sequencing gel was run vertically at 1500 volts at a constant power of 40W. After the run, gel plates were detached and the backplate with the gel bound to it was placed in wash mixture for 10min, followed by a wash in water. The plate was baked at 100°C in an oven for 20-30min until dry, cooled, and a Kodak X-ray film placed over it in a cassette. The autoradiogram was exposed and sequence read using the Staden GCG software.

Quick denaturation methods by glycol/heat or alkali were also used for sequencing along with the Sequenase™ Quick-Denature™ Plasmid Sequencing kit (USB). Glycerol-tolerant gel buffer was used for electrophoresis of glycerol denatured samples instead of TBE buffer owing to less distortion of bands. A 6% urea-PAGE gel was prepared as described above with 2.5ml of 20x glycerol tolerant buffer.

2.13.3C Direct Sequencing of PCR products

Some PCR products were directly analysed before cloning, using the Sequenase PCR product sequencing kit. 5µl of the gel purified PCR product (~30ng/µl required for sequencing a 1000bp product) was treated with 1µl each of enzymes exonuclease I (10U/µl) which removed residual single stranded DNA produced by PCR, and shrimp alkaline phosphatase (2U/µl) which removed any remaining dNTPs from the PCR mixture that may otherwise interfere with the labeling step.

The reaction mixture was incubated at 37°C for 15min and enzymes inactivated by heating to 80°C for 15min. 1µl of a gene specific primer (10 pmol/µl) was added to the treated PCR product and the volume made up to 10µl. The sample was heated for 3min at 100°C and cooled at once in an ice/water bath for 5min. 2µl of 5x sequencing reaction buffer was added and the tube used in the labeling reaction (Section 2.12.3A) and processed as described.

2.13.4 Automated Sequencing

Some DNA samples were sequenced on an automated ABI PRISM 377 DNA sequencer by the dye terminator method and cycle sequencing. Dideoxynucleotides labeled with rhodamine dye were used. DNA template to be sequenced was provided in a 0.5ml microfuge tube as double-stranded DNA (300-500ng) or as a PCR product (30-90ng) in a total reaction volume of 12µl with 3.2pico moles of a suitable primer. Cycle sequencing was carried out on a PTC-100 Programmable Thermal Controller; MJ Res., Inc.with a hot start at 96°C for 4min, followed by 30 cycles of 96°C, 30sec; 50°C, 20sec; 60°C, 4min. Samples were run on a sequencing gel (See 2.13.3B) prepared with electrophoresis grade Bio Rad reagents.

2.13.5 Sequence Analysis

Sequences were analysed on a Silicon Graphics Indy workstation using a GCG suite of programs (Genetics Computer Group, Madison, WI). Databases were searched using the TFASTA method (Devereux *et al.*, 1984) to compare the query sequence in all six reading frames with specific amino acid sequences (Pearson and Lipman, 1988).

2.14 Immunocytochemistry

2.14.1 Affinity column purification of antibody

REAGENTS	COMPONENTS
Hydrochloric acid	Commercially available as a 12M stock, pH 2 (Sp.Gr.=1.12)
Diethylamine	Commercially available as a 9.7M stock, pH 10.7 (Sp.Gr.= 0.7056, Mr = 73.14g)
Carb-chloride solution	0.1M $NaHCO_3$, 0.5M NaCl (pH 8.0)
Sodium acetate/citrate buffer	1M sodium acetate, pH 6.0; pH adjusted with 1M citric acid
Phosphate buffer	0.1M Na_2HPO_4, (pH 7.3); pH adjusted with 1M NaH_2PO_4
Preserve solution	20mM phosphate buffer (pH 7.3); 0.5M NaCl; 0.01% (w/v) azide

Selective purification of the specific antibody (Ab) from a mixture of Abs present in the antisera was carried out on an antigen (Ag) column. Abs were purified by affinity purification of a polyclonal serum. The Ag column was prepared by covalent coupling of a peptide (antigen) to a solid phase matrix, followed by addition of the antiserum. Specific Abs bound to the the Ag column and non-specific Abs were washed away. Abs were finally eluted from the column by high pH.

15mg of the peptide was dissolved overnight in 5ml of carb-chloride solution in a Falcon™ tube at 4°C in a rotator. 1gm of Sepharose 4B (CNBr activated) was suspended in 3.5ml of 1mM HCl (pH 2.0). Beads were allowed to swell for 10min, to ~5.0ml and then washed on a sintered funnel with 200ml each of 1mM HCl, dd water and 0.1M $NaHCO_3$. Washed, HCl treated Sepharose gel beads were added to the Falcon™ tube containing the dissolved peptide, and contents allowed to mix at 4°C in a rotator. Excess peptide was washed in three bed volumes (15ml) of carb-chloride solution. Remaining active groups were blocked overnight with 0.1M Tris-HCl (pH 8.0) at 4°C in a rotator. The ligand bound sepharose was finally washed in three cycles of alternating pH, with five bed volumes (25ml) of 0.1M sodium acetate/citrate buffer, (pH 4.0); 0.5M NaCl and 0.1M Tris-HCl, pH 8.0; 0.5M NaCl. The matrix was then stored in preserve solution until it was used to pack a column.

A 1ml syringe barrel was used as a column and packed with the matrix. The

column was connected to an Atto Perista Pump (Hertfordshire) and the gel matrix washed with 20mM phosphate buffer followed by a 20ml pulse of 0.5M of diethylamine. A second wash with 20mM phosphate buffer followed. The serum sample was applied to the column and allowed to recycle overnight at 4°C at 1ml/min. To carry out the elution process, the sample was allowed to pass through a Pharmacia LKB Pump P-1 with an optical UV unit. This was connected to a UV chart recorder to record changes in absorbance at 280nm at 1cm/min as the sample was passed through. The column was washed with 20mM phosphate buffer (pH 7.3) and Ab eluted with diethylamine (pH 10.7). The eluant was collected in a tube. The column was washed again in 20mM phosphate buffer, and stored in 20mM phosphate buffer, 0.01% azide or regenerated with alternating cycles of pH as described earlier, and finally stored in preserve solution.

The eluant collected from the affinity column was neutralised by dialysis. The sample was transferred to a dialysis tube. Dialysis tubing was prepared by boiling in dd water and then in 1mM EDTA (pH 8.0). Treated tubing was stored at 4°C in 1mM EDTA, 0.01% (w/v) azide and rinsed thoroughly in water before use. Dialysis was carried out overnight at 4°C against 5 litres of PBS with 2-3 changes. The sample was finally concentrated by placing the dialysis tube in PEG 20,000 until the volume reduced to 1-2ml. Ab specificity and titre were determined using ELISA tests.

2.14.2 ELISA

Serum antibody titre was measured by ELISA. In this technique, the antigen (peptide or protein) binds antiserum containing the anti-peptide antibody (Ab). This is allowed to bind a second Ab conjugated to peroxidase enzyme. The enzyme catalyses the formation of a coloured substance which can be quantified to indicate the amount of Ab present.

REAGENTS	COMPONENTS
Coating buffer	15mM Na_2CO_3; 35mM $NaHCO_3$, pH 9.6; 0.01% (v/v) NaN_3
Wash buffer	PBS, 0.1% (v/v) Tween
Blocking buffer	PBS, 0.1% (v/v) Tween, freshly prepared 1% (w/v) casein
Stock sodium acetate/citrate buffer	1M sodium acetate, pH 6.0 with 1M citric acid
Stock 3, 3′, 5, 5′ tetramethyl benzidine solution	10mg/ml TMB in DMSO
Substrate solution	5% (v/v) stock sodium acetate/citrate buffer, 15% (v/v) stock TMB solution, 0.006% (v/v) hydrogen peroxide (30% stock)
Stop solution	1.84M H_2SO_4 (1:10 dilution of 98% stock)

96 well microtitre plates were coated overnight with 100µl of 10µg/ml antigen in coating buffer. Plates were covered and incubated at 4°C. Unbound antigen was washed off the plates with wash buffer squirted from a wash bottle. The plates were drained of any residual solution by inverting and tapping the bottom on paper towels. This avoided cross contamination or any dilution. Adsorption sites of the wells were then blocked with 250µl blocking buffer for 2h at room temperature. Plates were again washed twice in wash buffer. Serum dilutions were applied at 100µl per well in wash buffer and plates incubated for 2h at room temperature or overnight at 4°C. Unbound primary Ab was removed by washing three times in wash buffer. 100µl of secondary Ab (anti-goat rabbit peroxidase conjugate) at a 1:1000 dilution in wash buffer was applied to each well. Plates were incubated at room temperature for 2h and washed again three times in wash buffer, followed by two washes in PBS. 100µl substrate solution was applied to each well and plates left at room temperature for 15min. The colour reaction was terminated with 50µl stop solution and O.D read in a Multiskan® MCC/340, Labsystems ELISA plate reader at 450nm. Antibody titre was determined by plotting a graph between 1/serum dilution and absorbance on X and Y co-ordinates respectively. The inflection point of the titre of the post-immune graph when interpolated by drawing a line to the X-axis resulted in the Ab titre.

(C) cDNA library construction and screening of *Ascaris suum*

2.15.1 First and second strand synthesis

Total RNA was isolated from 2g *Ascaris suum* heads and suspended in 0.5ml of 0.5% (w/v) SDS. Poly-[A]$^+$ RNA was purified from 400µg total RNA and suspended in 10µl of DEPC water (Section 2.9). Subsequent reactions for library construction were carried out using reagents provided in the ZAP-cDNA® Synthesis Gigapack® III Gold Cloning kit (Stratagene). First strand cDNA synthesis was carried out with

5µg	poly-[A]$^+$ RNA
2.0µl	linker-primer (1.4µg/µl)
5µl	10x first strand buffer
3.0µl	first-strand methyl nucleotide mixture [10mM dATP;dGTP;dTTP;5mM 5-methyl-dCTP]
1µl	RNase Block Ribonuclease inhibitor (40U/µl)

in a total volume made up to 48.5µl with DEPC-treated water. The tube was left at room temperature for 10min to allow template-primer annealing. 1.5µl *M-MuLVRT* (50U/µl) was added and the tube gently vortexed and incubated at 37°C for 1h

Second strand synthesis was carried out at 16°C for 2 1/2h. To 47µl of the first strand reaction were added

20µl	10x second strand buffer
6µl	second strand nucleotide mixture [10mM dATP, dGTP, dTTP; 26mM dCTP]
106.2µl	DEPC-water
3.5µl	*RNase H* (0.9U/µl)
11.0µl	*DNA polymerase I* (9.1U/µl)

2.15.2 Blunt-ended cDNA termini and modification

cDNA termini were blunt-ended by the addition of

23µl	blunting dNTP mix
2µl	cloned *Pfu DNA polymerase* (2U/µl) and incubating the reaction at 72°C for 30min. The reaction was extracted with phenol:chloroform and DNA ethanol

precipitated. The DNA pellet was suspended in 9 µl of *EcoRI* adapters and left for 15min. Blunted cDNA ends were ligated at 4°C for 48h, with 1µl each of 10x ligase buffer, 10mM rATP and of *T4 DNA ligase* (4U/µl). The ligase was inactivated by heating the tube to 70°C in a water bath for 30min. *EcoRI* adapters were phosphorylated at 37°C for 30min in

1µl 10x ligase buffer

2µl 10mM ATP

6µl dd water

1µl *T4 polynucleotide kinase* (10U/µl). The kinase was heat inactivated at 70°C for 30min. 28µl of *XhoI* buffer and 3µl of *XhoI* (40U/µl) were added and the cDNA digested at 37°C for 1.5h. 5µl of 10x STE buffer was added and the cDNA size fractionated.

2.15.3 Size Fractionation

A Sephacryl S-500 spin column was used for size selection of cDNA. A 1ml plastic syringe loaded with Sephacryl, pre-equilibrated with 1x STE buffer [1M NaCl; 200mM Tris-HCl (pH 7.5); 100mM EDTA] was loaded with 60µl of the sample in 1x STE. The column was centrifuged at 400 x *g* for 2min, and the eluant collected in a sterile microfuge tube (fraction 1). This was repeated again, and if required, a third time (fractions 2 and 3). All fractions were extracted with phenol:chloroform (1:1) in order to remove the kinase, thus preventing religation of the vector and *XhoI-EcoRI* vector fragments and a high blue background upon plating. The cDNA was ethanol precipitated and pellet dissolved in 5µl of dd water. An ethidium bromide assay was carried out to estimate the concentration of DNA in the three fractions.

2.15.4 Ligation and Packaging of Lambda Zap recombinants

~100ng of resuspended cDNA was ligated into 1µl Uni-ZAP™ XR vector (1µg/µl) in the presence of 0.5µl each of 10x ligase buffer and 10mM rATP (pH 7.5) and 0.5µl of *T4 DNA ligase* (4U/µl)in a total volume of 5µl at 4°C for 48h. 1µl of the ligation mix was packaged into Gigapack III Gold packaging extract. A test ligation to ligate

pBR322 test insert */SalI* (compatible with *XhoI*) */EcoRI*, into Uni-ZAP XR vector was carried out in parallel.

The ligation mix containing DNA was added to a thawed freeze/thaw packaging extract. The tube was pulse spun and incubated at room temperature for 2h. 500µl of SM buffer and 20µl of chloroform were added and the tube spun briefly to sediment any debris. The supernatant containing the phage was transferred to a fresh tube and stored at 4°C before determining the phage titre in the host strain, *E.coli* XL1-Blue MRF'. A positive control for packaging ~ 0.2 µg of the wild type λ*c*I857 *Sam*7 lambda control DNA in the host strain VCS257 was run in parallel. The growth conditions of the host strains used are shown below.

Host Strain	**Medium for bacterial**	**Medium for bacterial cultures for phage titration (final conc.)**	**Special Features**
XL 1-Blue MRF' (*E.coli* host strain)	LB-tetracycline	LB with 0.2% (w/v) maltose - 10 mM $MgSO_4$	*RecA*$^-$, does not require *supF* genotype, produces blue plaques with non-recombinants and white plaques with recombinants.
VCS257 (control host strain)	LB	LB with 0.2% (w/v) maltose - 10 mM $MgSO_4$	Derivative of DP50 *supF*. Control test DNA, λ*c*I857 *Sam*7 requires the *supF* mutation.

2.15.5 Plating and Titration

1µl of a 1:10 diluted packaging reaction in SM buffer (100mM NaCl; 8mM $MgCl_2$; 50mM Tris-HCl, pH 7.6) was added to 200µl of XL1-Blue cells at OD^{600} = 0.5. In parallel, 10µl of a 10^{-4} dilution of the control packaging reaction was added to 200µl of the VCS257 host strain with OD^{600}=0.5. Phages were allowed to adsorb cells for 15min at 37°C and then added to 3ml NZY top agar at 48°C with 15µl of 0.5M IPTG in water and 50µl of 250 mg/ml X-gal in DMF and poured on 150mm NZY agar plates. Plates were inverted and incubated overnight at 37°C.

2.15.6 Single clone excision

DNA inserts were isolated by using *in vivo* excision properties of the Lambda Zap bacteriophage (Short *et al.*, 1988). Cored plaques were vortexed in 500µl of SM buffer and 20µl of chloroform, and incubated o/n at 4°C. 200µl XL1-Blue cells at $O.D^{600}$ = 1.0, 250µl of phage stock (containing > 1 x 10^5 phage particles) and 1µl of ExAssist helper phage (>1 x 10^6 pfu/ml) were combined in a 50ml polypropylene tube and incubated at 37°C for 15min. 3ml of LB was added and cells shaken at 37°C for 3h. The tubes were then heated at 70°C for 20min to inactivate parental phages and kill the bacteria. Debris was spun at 1000 x *g* for 15min. The supernatant containing excised pBluescript phagemid was packaged as filamentous phage particles and stored at 4°C. Excised phagemids were plated by mixing , 200µl of SOLR cells at OD^{600}=1.0 with 100µl of the phage supernatant and incubation at 37°C for 15min. 200µl of the mixture was plated on LB/ampicillin plates and incubated o/n at 37°C. Single colonies containing pBluescript double stranded phagemid with the cloned DNA insert were purified on new LB/ampicillin plates.

2.15.7 Library amplification and screening

The library was amplified in order to obtain a large and stable quantity of a high titre stock. 600µl of XL1-blue cells at OD^{600}=0.5, diluted in 10mM $MgSO_4$, was mixed with 56µl of library suspension containing ~50,000 plaque forming units (pfu), and aliquoted into each polypropylene tube and incubated at 37°C for 15min. 6.5ml of molten NZY top agar (48°C) was added to each aliquot and poured onto prewarmed NZY 150mm plates of bottom agar. Plates were incubated at 37°C for 5-8h, until plaques were ~0.5mm in diameter and just sub-confluent. 10ml of SM buffer was pipetted onto each plate and incubated at 4°C o/n with gentle shaking. The SM buffer was then pooled from the plates into 50ml polypropylene (Falcon) tubes, the top agar scraped off, and added to the buffer. Chloroform was added to a final concentration of 5% (v/v), the contents mixed, and tubes incubated at 4°C overnight with gentle shaking. Top agar and cell debris were removed by centrifugation for 10min at 4000 x *g*. The supernatant was transferred to a sterile tube and chloroform added to final concentration of 0.3% (v/v). The amplified

library was titred on *E.coli* XL1-Blue cells and stored at 4°C.

The amplified library was screened by plaque lifts of the same, on to Hybond™ nylon membranes placed gently on top of the agar and left for 1min to allow for transfer. The corresponding orientation was marked for by picking through the agar using a needle. The plaque blotted membrane was placed colony side up on a pad of absorbant filter paper soaked in denaturing and neutralising solutions (See Section 2.13) for 7min and 3min respectively and finally in 2x SSC. Duplicate lifts were carried out in parallel. The replica membrane was not allowed to move once placed on the agar surface. The same orientation was marked for as done previously. All membranes were air dried colony side up and baked at 80°C for 2h. Labeling using a DNA probe for hybridization was carried out as described in Section 2.9.6E. Membranes were washed twice in 50 ml of 2x SSC, 0.1% (w/v) SDS for 10 min at room temperature followed by 50ml of 1x SSC, 0.1% (w/v) SDS at 65°C for 20min. A final wash with 0.1x SSC, 0.1%(w/v) SDS for 10min at 65°C was carried out if required. Membranes were air dried briefly, wrapped in clingfilm and exposed overnight to a Kodak X-Ray film in a cassette with intensifying screens at –70°C. In case of any indications of a positive signal during primary screening, the film was aligned with the needle holes made on the plate and corresponding regions cored with a flat end of a sterile glass Pasteur pipette. The cored agar was transferred to a tube containing 1ml of SM buffer and 20µl chloroform. The tube was vortexed and phage titre determined on small 100mm∅ NZY agar plates containing ~50 plaques (each cored plaque has ~10^6 pfu). Secondary screening of plaque lifts was then carried out as was done with primary screening.

(D) Immunocytochemical staining in *H. contortus* and *A. suum*

REAGENTS	COMPONENTS
Antibody dilution solution	0.1% (w/v) BSA, 0.5% (v/v) Triton X-100, 0.05% (w/v), 0.05% (v/v) NaN_3 in PBS
Artificial Perenteric Fluid	
BME solution	5% (v/v) 2-mercaptoethanol; 1% (v/v) Triton X-100; 125mM Tris, pH 6.9
Collagenase buffer	1mM $CaCl_2$; 100mM Tris-HCl, pH 7.5
Fix solution	4% (v/v) Paraformaldehyde (PFA) in PBS (Section 2.5)
Storage buffer	0.1% PFA in PBS
Wash solution 1	0.1% (v/v) Triton X-100 in PBS
Wash solution 2	0.5% (v/v) Triton X-100 in PBS

2.16.1 *H. contortus* whole worm preparations

Adult *H. contortus* in fix solution for 12h were washed 3x in PBS and stored at 4°C in storage buffer (See Section 2.7.1B). Worms were washed in PBS and permeabilised by incubating overnight in BME solution at 37°C in a water bath in a Falcon™ tube. The worms were transferred to a fresh tube and treated with 115 collagen digestion units/ml of collagenase in collagenase buffer for 8 to 16h at 37°C in a shaker at 110rpm. After digestion of the cuticle, worms were incubated in different dilutions of the primary Ab in Ab dilution solution at 4°C for 72h. Unbound Ab was removed in three PBS washes and the worms transferred to a fresh tube to avoid any cross-contamination with the secondary Ab. A 1:200 dilution of a TRIT-C conjugated anti rabbit IgG secondary serum in Ab dilution solution was added and the worms incubated overnight at 4°C. Unbound IgG was removed with three 15min washes in PBS, followed by an overnight wash in wash solution 1. The worms were mounted on slides using Vectashield® mounting medium and examined under a Olympus BHS fluorescence microscope in the green excitation region with exciter filter G (IF-545+BG-36), dichroic mirror in the range (580+0.-590) sand barrier filter R-610. Alternately, a Zeiss LSM 510 confocal fitted to an inverted Axiovert 100M confocal microscope, using a helium neon

laser and rhodamine filter set was used. Negative controls for immunostaining were set up in parallel in (1) the absence of primary Ab and with (2) presence of the peptide. The peptide was added to the primary Ab in Ab dilution solution to a concentration of 20μg/ml. The dilutions were incubated at 37°C for 1h and processed at once.

2.16.2 *A. suum* cryosections

Fixed *A. suum* (see section 2.7.2) were washed briefly in PBS and dissected ~3cm from the anterior end to obtain the heads. The specimen was placed in the centre of a parafilm walled chuck, a specimen holder for cryostats. The chuck was placed on a beaker with dry ice and OCT compound added to the capsule. The specimen was held in position in the centre as the OCT compound was allowed to freeze. The parafilm around the chuck was then removed and the set up mounted on a cryostat. 20μm thick sections were obtained and collected on Vectabond treated slides. Cryosections were allowed to freeze dry at −20°C for at least 24h and thawed for 20min at room temperature before use.

REAGENTS	COMPONENTS
OCT compound	Commercially available as a mixture of polyvinyl alcohol, PEG and dimethyl benzyl ammonium chloride
Vectabond™ reagent slide treatment	Slides rinsed in acetone, followed by Vectabond™ reagent in 350ml acetone for 5min and washes in acetone dd water. Slides dried at 40°C-50°C
Vectashield® mounting medium	Commercially available

Sections were circled with a DAKO pen for immunohistochemistry which contained any liquid medium when added. Cryosections were washed briefly in PBS and drained off it to add the primary Ab in a 1:5 dilution in Ab dilution solution. Slides were left for 2h at room temperature and then washed in PBS three times before addition of a 1:200 dilution of a TRIT-C conjugated anti rabbit IgG secondary serum in Ab dilution solution. The slides were incubated overnight at 4°C and then washed in PBS before mounting in Vectashield mounting medium and visualising under the confocal microscope.

2.16.3 *A. suum* whole worm preparations

Live *A. suum* were maintained in artificial perienteric fluid at 37°C for a maximum of five days , prior to injection with 4mg/ml collagenase in PBS. The worm was ligatured ~3cm from the anterior end and also in the region where the ovaries appeared to begin, thus exposing a restricted portion of the worm to collagenase. The amount injected varied between 250-500µl depending upon the size of the worm (Johnson and Stretton, 1987). After incubation at 37°C for 90min, the worms were dissected at the ligatured ends and cut along the lateral line. The collagenase treatment dissociates muscle cells which separate from the body wall when washed with PBS using a Pasteur pipette. The remaining body wall was carefully washed off dissociated tissues to expose the cuticle and hypodermal layer, with neurons embedded on it. Both muscle cell and body wall preparations were fixed for 12h at 4°C in PFA fix solution. Fixed tissue was washed 3x in PBS and stored at 4°C in storage buffer.

Muscle cells or whole mount preparations were washed in PBS and incubated at 4°C in eppendorf tubes in dilutions of primary antiserum in Ab dilution solution for 40h. Unbound primary Ab was removed with 3 x 15min washes in wash solution 2. Samples were incubated in 1:500 dilution of TRIT-C conjugated anti rabbit IgG secondary serum in Ab dilution solution for 5h. Samples were then washed 3 x 15min in wash solution 2, mounted, and examined as described in Section 2.16.1.

(3) CLONING AND CHARACTERIZATION OF RECEPTOR SUBUNITS ENCODED BY AN ALTERNATIVELY SPLICED GENE FROM *HAEMONCHUS CONTORTUS*

3.1 Introduction

The RACE-PCR (Rapid Amplification of cDNA Ends) technique serves as a rapid and sensitive *in vitro* procedure of detecting rare transcripts. This has proved particularly useful when looking for receptor subunit cDNAs forming a rare heterogeneous pool. The mRNA reverse transcription step can be modified and repeated until the desired cDNA is produced. In addition, information can be obtained about alternative splicing, thus allowing for separation of cDNAs produced from different transcripts prior to cloning.

Degenerate primers were used on cDNA reverse transcribed from mRNA isolated from a mixed egg population of *Haemonchus contortus*. This resulted in the amplification of several partial receptor subunit cDNAs, *HG1-5* (Laughton *et. al.*, 1994). The RACE-PCR technique was used to obtain 3′ and 5′ end sequences of one of the partial cDNAs denoted *HG2/3*. It shows high homology to the alternatively spliced *gbr-2* gene from *C.elegans* encoding two glutamate-gated chloride (GluCl) ion channel receptor subunits, GBR-2A and 2B. The *Ce-gbr-2a* cDNA includes a large 3′ untranslated region (UTR) within which is an open reading frame that would encode the COOH-terminal portion of a second inhibitory subunit. Both subunits thus have a common NH_2-terminal domain but two different transmembrane domains (Laughton *et. al.*, 1997).

Attempts were made to obtain the full-length sequence of *HG2/3* by PCR and determine if it was encoded by an alternatively spliced gene. PCR amplification of genomic DNA across the transmembrane/extracellular boundaries could show if it corresponded with intron/splice sites and confirm if the gene was alternatively spliced. The HG2/3 sequence was also examined from ivermectin (IVR) resistant population of *H. contortus* (Section 2.7.1A) and compared with cDNAs from IVR susceptible isolates in order to identify putative resistance associated mutations if any.

3.1.1 PCR techniques

Genomic DNA amplificatons were carried out by touchdown PCR. This uses conditions slightly modified from the standard PCR method by allowing for enhanced specificity during amplification, especially of G-C rich sequences (Don, 1991). Mispriming resulting in spurious smaller bands are largely avoided by a touchdown of annealing temperatures. PCR reactions are carried out by starting at or above the expected annealing temperature, followed by a stepwise decrease to an annealing temperature. This results in specific amplification to start with and non-specific amplification thereafter, thus enriching the target cDNA (Osterrieder, 1994).

3.1.2 PCR Quantitation

A 'semi-quantitative' PCR approach was made to determine the relative expression levels of *gbr-2A* and *gbr-2B* mRNAs in IVR susceptible and resistant *H. contortus* eggs. The amount of a gene specific transcript can be measured by estimating the intensity of a PCR amplified band on an ethidium bromide stained agarose gel, or by densitometry of an X-ray film (Wang *et al.*, 1989). Although the method of quantitation may not be as accurate as other time-consuming methods such as northern blotting and RNase protection assays, careful optimisation of the possible variable parameters makes the PCR approach a simple and sensitive means of analysis (Hengen, 1995).

3.2 Results

3.2.1 Full length amplification of *Hc-gbr-2A* and *Hc-gbr-2B*

3.2.1.1 Template

Total RNA was isolated from 0.9g of *H. contortus* eggs using the Chomczynski method and suspended in 0.5ml of 0.5% SDS. The yield was approximately 0.5mg as estimated by agarose gel electrophoresis. 2µl was run on a 1% (w/v) agarose gel (Figure 3.1). Poly $[A]^+$ RNA was isolated from 200µg of total RNA and reverse transcribed to cDNA using primer RoRidT and used in PCR reactions (Section 2.9.1-4).

3.2.1.2 PCR of *Hc-gbr-2a and Hc-gbr-2b* cDNA

1µl of a 1:5 dilution of the cDNA was used as template to carry out PCR reactions using the High Fidelity Expand™ System on a PTC-100, (Programmable Thermal Controller); M J Res. Inc. PCR machine. Primers were designed as described in section 2.11.1. Sense primer 5HGFULL and antisense primer 3HGFULL (Table 3.1) corresponding with start and stop codons of the *Hc-gbr-2* cDNA and incorporating restriction sites *XbaI* and *HindIII* respectively in their linker adaptors to facilitate cloning, were used for amplification. The reaction conditions were a 2min hot start at 94°C, followed by 35 cycles of 94°C, 15sec; 65°C, 30sec; 72°C, 1min and a final extension of at 72°C for 7min, with a final concentration of 1.5mM Mg^{+2} cations. Two bands of sizes 1.3Kbp and 2.0Kbp were noted on a 1% (w/v) agarose gel (Figure 3.2). The smaller sized DNA was subcloned into pBluescript and sequenced. The larger sized PCR product was sequenced directly as it was present in low amounts.

3.2.1.3 Subcloning of *Hc-gbr-2b* cDNA

The smaller sized band was excised from the gel, DNA purified using Sephaglas™ kit (Section 2.10.2), and 2µl used in PCR reamplification under the same conditions as described above. The PCR product was run on a 1% (w/v) gel, Southern blotted, and hybridized to an internal oligonucleotide primer 3HG3PR1 (Section 2.13) (Figure 3.3).

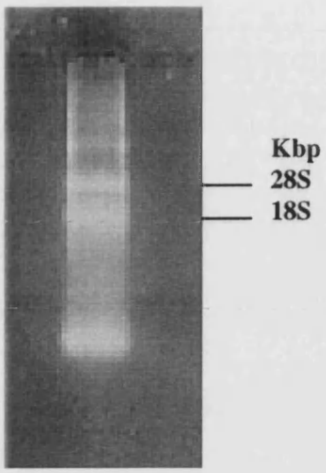


Figure 3.1 Total RNA extracted from *H. contortus* eggs run on a 1% (w/v) agarose gel in TBE.

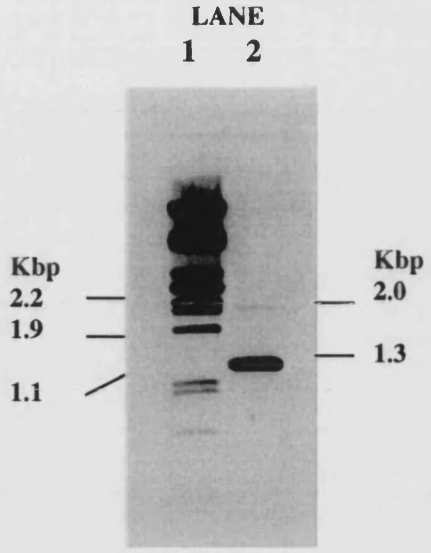
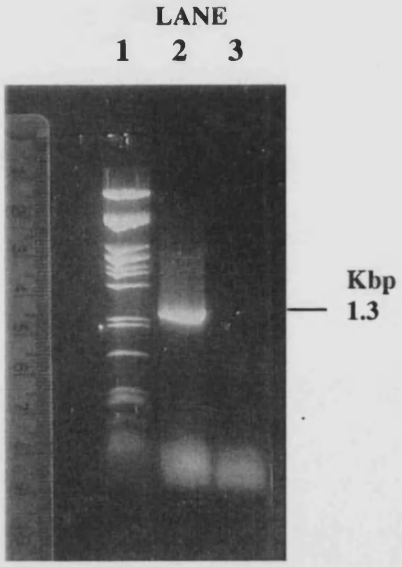


Figure 3.2 Full length amplification of *Hc-gbr-2* cDNA from *H. contortus* eggs. 1% (w/v) agarose gel with TBE with **Lane1**: λ*PstI* marker; **Lane2**: PCR amplification product.



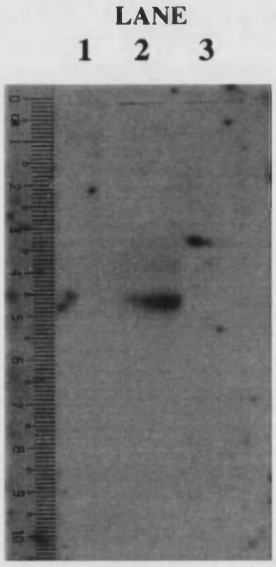


Figure 3.3 Southern blot of a 1% (w/v) agarose gel electrophoressed with the *Hc-gbr-2b* PCR amplification product from *H. contortus* eggs. **Lane 1**: λ*PstI* marker; **Lane2**: PCR product; **Lane 3**: No template PCR control.

Amplified DNA was purified from the PCR mix using the Wizard™ PCR Preps DNA Purification System (Section 2.10.2C) (Figure 3.4A), digested with enzymes *XbaI* and *Hind III* and phenol-chloroform extracted (Section 2.10.3-4). This was subcloned into pBluescript SK^+ vector processed in a similar fashion (Figure 3.4B). Eleven resulting clones were checked by restriction digests (Figure 3.5). Two clones, pH2BSJ2.1 and pH2BSJ2.7 with the right size of insert were sequenced in both directions using pBluescript sense T3 and antisense M13 primers on an automated sequencer.

Primer	Sequence	Tm (°C)	Peptide Motif
5HGFULL	5′ ATCGTT*AAGCTT*GGCATGCGCAATTCCGTCCCTC 3′	72	MRNSVP
3HGFULL	5′ TGATCG*TCTAGA*TGGTCAGTCGAGGTTGCTTTG 3′	76	QSNLD
5HG2PR1	5′ GGTGTTCCCTATTGGATATGCC 3′	66	VFPIGYA
5HG2PR2	5′ GCTCGCAAGGATATGAGTTGC 3′	64	ARKDMSC
5HG2PR3	5′ ATGGTGCTACGTCGAGAGTTC 3′	64	MVLRREF
5HG3PR1	5′ GGCCGGAAGGAATTTCTTCGG 3′	66	GRKEFLR
5HG3PR2	5′ AGTTGTGCTCGGGTCAAACTTC 3′	68	SCARVKL
3HG2PR1	5′ GCATATCCAATAGGGAACACC 3′	62	VFPIGYA
3HG3PR1	5′ GGATGAGGTAGTAACTGTACTC 3′)	64	EYSYYLIQ
3HG3PR2	5′ ATACGCATTTCGACCCAACATATC 3′	68	DMLGRNAY
5HCL9	5′ ATGAAGCTGGTCGAGTCCAATG 3′	66	MKLVESN
3TSA2	5’ GGATCGAGCCGTCCTTGTGAA 3’	66	FTRTLD
SL1	5′ GGTTTAATTACCCAAGTTTGAG 3′	60	
RoRidT	5′ GACTACGTTAGCATCTAGAATTCTCGAG $[T]_{17}$ 3′		
Ceg2S1	5′ CTGGAGACAACATCAATTCGTT 3′	62	RYKERSK
Ceg3QA1	5′ GCAAAACGACCCGTGCACAAC 3′	66	CARVVL

Table 3.1 Primers used in PCR amplification of *H.contortus* and *C.elegans* cDNA. Adapter sequences are underlined, and the sequence corresponding to restriction sites shown in italics.

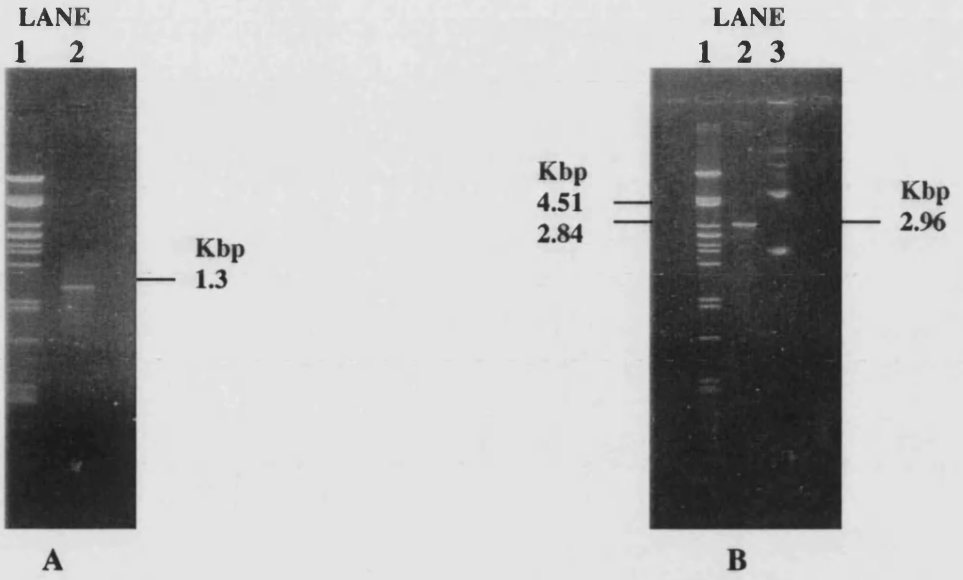


Figure 3.4 *XbaI / HindIII* digested and phenol chloroform (1:1) extracted samples of **(A)** PCR amplified *Hc-gbr-2b* cDNA and **(B)** pBluescript DNA. 1% (w/v) agarose gel in TBE with **(A) Lane1**: λ*PstI* DNA marker**; Lane2**: PCR product and **(B) Lane1**: λ*PstI* DNA marker; **Lane2**: enzyme digested pBluescript DNA; **Lane3**: undigested pBluescript DNA.

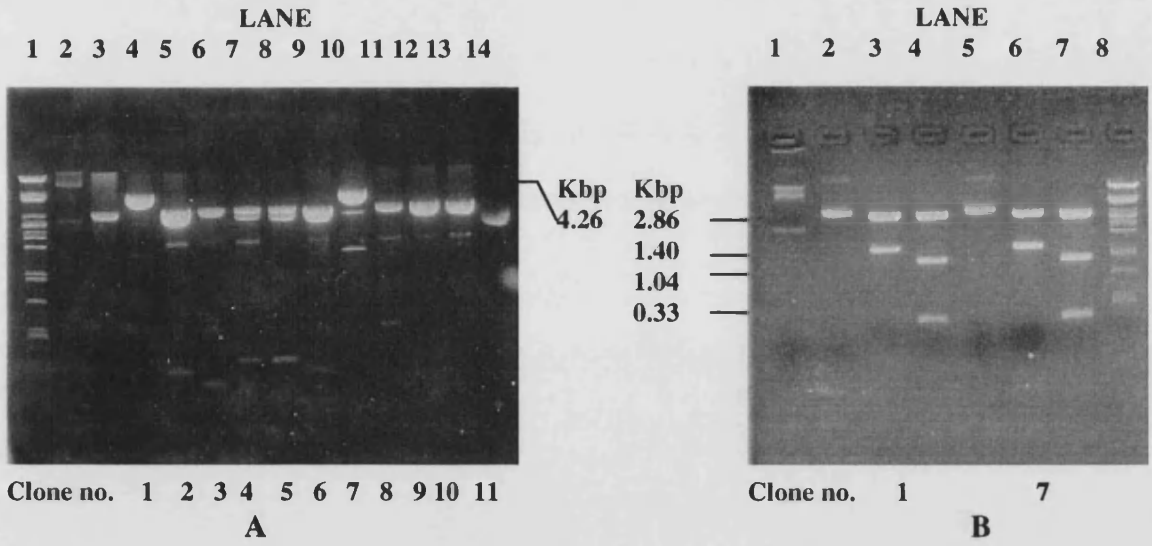


Figure 3.5 Restriction digests of plasmid DNA from clones of *Hc-gbr-2* in pBluescript SK^+ vector. 1% (w/v) agarose gel in TBE with **(A)** *HindIII* digested DNA. **Lane1**: λ/*PstI* DNA marker; **Lane2**: undigested pBluescript DNA; **Lane3**: enzyme digested pBluescript DNA; **Lanes4-14**: enzyme digested plasmid DNA of clones no. 1 to 11. **(B)** Restriction digests of clones no. 1 and 7 respectively. **Lane1**: undigested pBluescript DNA; **Lanes2, 5**: undigested DNA; **Lanes 3, 6**: *XbaI / HindIII* digested DNA; **Lanes4, 7**: *SacI / KpnI* digested DNA; **Lane8**: λ/*PstI* DNA marker.

3.2.1.4 Sequence Analysis

The sequence of the larger 2.0 Kbp band corresponds to the full length *Hc-gbr-2a* cDNA. It encodes a polypeptide of 421 amino acids with a predicted molecular weight of 48,597 for the unglycosylated protein (Figure 3.6A, B). The smaller 1.3Kbp band corresponds to the alternatively spliced *Hc-gbr-2b* mRNA. The nucleotide and derived amino acid sequence of this alternatively spliced cDNA cloned in pBluescript is shown in Figure 3.7. It encodes a polypeptide 439 amino acids long with a predicted molecular weight of 50,690 for the unglycosylated protein (Figure 3.6C). The sequence of both *Hc-gbr-2b* clones was identical, lacking nucleotides 736 to 1409 from the full length *Hc-gbr-2a* transcript. Sequences of the polypeptides encoded by the two cDNAs exhibited all the features of a inhibitory ligand-gated ion channel receptor subunit- an extracellular NH_2-terminal domain containing four cysteine residues, two of which form a disulphide bond, a COOH-terminal channel forming domain with four transmembrane spanning regions and an intracellular loop between the third and the fourth membrane spanning domains, containing potential sites for phosphorylation (Table 3.2). A comparison of the predicted amino acid sequences of the two cDNAs using the GCG program PILEUP confirmed the presence of a common NH_2-terminal domain with a site for N-linked glycosylation at asparagine residue 57 (coloured same in Figures 3.6 and 3.7) (Devereux *et. al.*, 1984). However, 57% identity is noted in the channel encoding domains of the two subunits. The TM II domains of the two subunits are very similar except for two differences in the Hc-GBR-2A subunit; a Thr→Ser substitution in position 295 and a Ser→Ala substituion in position 308 (Table 3.3). A table of comparison of the TM IV domains is also shown (Table 3.4).

The *Hc-gbr-2a* cDNA sequence was similar to the partial HG2/3 clone obtained by Laughton *et. al.*, (1994) except for two amino acid changes in positions 415 (Tyr→Phe) and 1888 (Asp→Tyr) (Figure 3.8). EMBL database search using the GCG-TFASTA program for the closest sequence matches to *Hc-gbr-2* showed highest identity with the alternatively spliced *gbr-2* gene from *C.elegans* encoding for subunits GBR-2A and 2B (Devereux *et. al*, 1984). A PILEUP of GBR-2A and 2B subunits from *H. contortus* and *C. elegans* is shown in Appendix II.

Figure 3.6A Derived peptide sequence of *Hc-gbr-2a* cDNA isolated from *H. contortus* eggs. Common NH_2-terminal domain is shown in red. Channel encoding domains of Hc-GBR-2A and 2B subunits are shown in blue and black respectively. The intron region is shown in lower case.

Figure 3.6 Dervied peptide sequences of alternatively spliced subunits **(B)** Hc-GBR-2A and **(C)** Hc-GBR-2B from *H. contortus*. The common NH_2- terminal is shown in red. The signal peptide cleavage sites in indicated by an arrow, the N-linked glycosylation site marked with an asterisk, and the cysteine residues underlined. Possible protein kinase C phosphorylation sites are shown by • while cAMP-dependent phosphorylation site is shown with a ♠.

MRNSVPLATR IGPMLALICT VSTIMSAVEA KRKLKEQEII QRILNNYDWR

VRPRGLNASW PDTGGPVLVT VNIYLRSISK IDDVNMEYSA QFTFREEWVD

ARLAYGRFED ESTEVPPFVV LATSENADQS QQIWMPDTFF QNEKVARRHL

IDKPNVLIRI HKDGSILYSV RLSLVLSCPM SLEFYPLDRQ NCLIDLASYG

YTTQDIKYEW KEQNPVQQKD GLRQSLPSFE LQDVVTKYCT SKTNTGEYSC

LRTQMVLRRE FSYYLLQLYI PSFMLVIVSW VSFWLDKDSV PARVTLGVTT

TM I

LLTMTTQSSG INANVPPVSY TKAIDVWIGV CLAFIFGALL EFAWVNYAAR

TM II *TM III*

KDMSCGQRMM KQLPQDGYRP LAGSQPRTSF CCRIFVRRYK ERSKRIDVVS

RLVFPIGYAC FNVLYWAVYL M*agcatggagcgtgctctactcgttcgtccg

TM IV

tccgtccgtccgtcctcattgtacctccgaactcacctcgtctcacttttcttc

tctcaattcttcgcctcgttgctttccatctcacccagtgccttattagtcgtt

tatagGEYSC ARVKLLLRRE YSYYLIQLYIP CIMLLVVSW VSFWLDKDAV

TM I

PARVSLGVTT LLTMTTQASG INSKLPPVSY IKVQAVDVWI GVCLAFIFGA

TM II *TM III*

LLEYAVVNYY GRKEFLRKEK KKKTRLDDCV CPSERPALRL DLSNYRRRGW

TPLNRLLDML GRNAYLSRRV DLMSRITFPS LFTAFLVFYY SVYVKQSNLD

TM IV

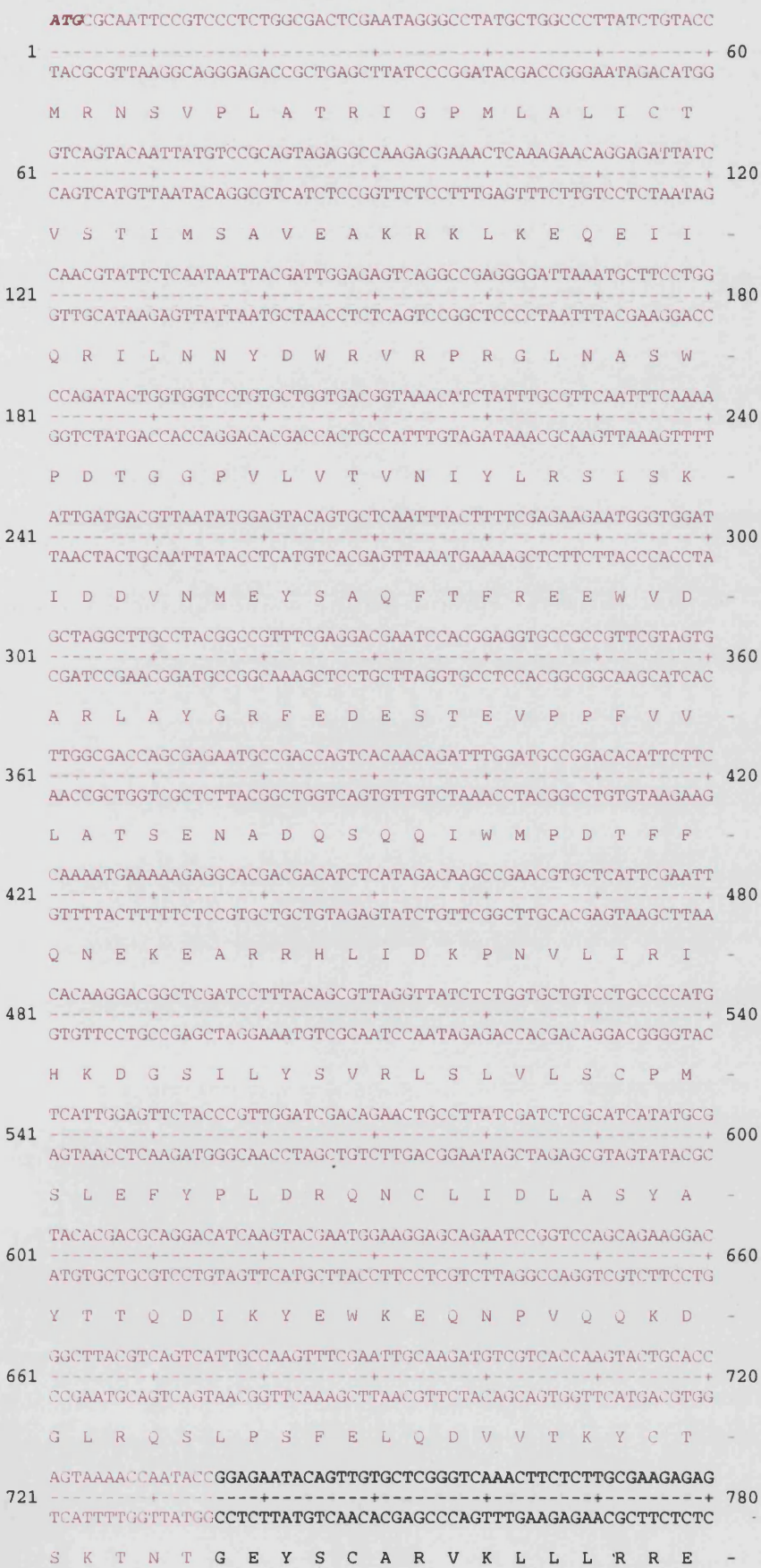
(A)

```
                                    ⇓
  1 MRNSVPLATR IGPMLALICT VSTIMSAVEA KRKLKEQEII QRILNNYDWR
      *
 51 VRPRGLNASW PDTGGPVLVT VNIYLRSISK IDDVNMEYSA QFTFREEWVD
101 ARLAYGRFED ESTEVPPFVV LATSENADQS QQIWMPDTFF QNEKVARRHL
151 IDKPNVLIRI HKDGSILYSV RLSLVLSCPM SLEFYPLDRQ NCLIDLASYA
201 YTTQDIKYEW KEQNPVQQKD GLRQSLPSFE LQDVVTKYCT SKTNTGEYSC
251 LRTQMVLRRE FSYYLLQLYI PSFMLVIVSW VSFWLDKDSV PARVTLGVTT
301 LLTMTTQSSG INANVPPVSY TKAIDVWIGV CLAFIFGALL EFAWVNYAAR
                                          •
351 KDMSCGQRMM KQLPQDGYRP LAGSQPRTSF CCRIFVRRYK ERSKRIDVVS
401 RLVFPIGYAC FNVLYWVYLM
```

(B)

```
                                    ⇓
  1 MRNSVPLATR IGPMLALICT VSTIMSAVEA KRKLKEQEII QRILNNYDWR
      *
 51 VRPRGLNASW PDTGGPVLVT VNIYLRSISK IDDVNMEYSA HFTFREEWVD
101 ARLAYGRFED ESTEVPPFVV LATSENADQS QQIWMPDTFF QNEKEARRHL
151 IDKPNVLIRI HKDGSILYSV RLSLVLSCPM SLEFYPLDRQ NCLIDLASYA
201 YTTQDIKYEW KEQNPVQQKD GLRQSLPSFE LQDVVTKYCT SKTNTGEYSC
251 ARVKLLLRRE YSYYLIQLYI PCIMLLVVSW VSFWLDKDAV PARVSLGVTT
301 LLTMTTQASG INSKLPPVSY IKAVDVWIGV CLAFIFGALL EYAVVNYYGR
          ♠                ●
351 KEFLRKEKKK KTRLDDCVCP SERPALRLDL SNYRRRGWTP LNRLLDMLGR
    ●
401 NADLSRRVDL MSRITFPSLF TAFLVFYYSV YVKQSNLD
```

(C)


ATGCGCAATTCCGTCCCTCTGGCGACTCGAATAGGGCCTATGCTGGCCCTTATCTGTACC
1
60
TACGCGTTAAGGCAGGGAGACCGCTGAGCTTATCCCGGATACGACCGGGAATAGACATGG
M R N S V P L A T R I G P M L A L I C T
GTCAGTACAATTATGTCCGCAGTAGAGGCCAAGAGGAAACTCAAAGAACAGGAGATTATC
61
120
CAGTCATGTTAATACAGGCGTCATCTCCGGTTCTCCTTTGAGTTTCTTGTCCTCTAATAG
V S T I M S A V E A K R K L K E Q E I I -
CAACGTATTCTCAATAATTACGATTGGAGAGTCAGGCCGAGGGGATTAAATGCTTCCTGG
121
180
GTTGCATAAGAGTTATTAATGCTAACCTCTCAGTCCGGCTCCCCTAATTTACGAAGGACC
Q R I L N N Y D W R V R P R G L N A S W -
CCAGATACTGGTGGTCCTGTGCTGGTGACGGTAAACATCTATTTGCGTTCAATTTCAAAA
181
240
GGTCTATGACCACCAGGACACGACCACTGCCATTTGTAGATAAACGCAAGTTAAAGTTTT
P D T G G P V L V T V N I Y L R S I S K -
ATTGATGACGTTAATATGGAGTACAGTGCTCAATTTACTTTTCGAGAAGAATGGGTGGAT
241
300
TAACTACTGCAATTATACCTCATGTCACGAGTTAAATGAAAAGCTCTTCTTACCCACCTA
I D D V N M E Y S A Q F T F R E E W V D -
GCTAGGCTTGCCTACGGCCGTTTCGAGGACGAATCCACGGAGGTGCCGCCGTTCGTAGTG
301
360
CGATCCGAACGGATGCCGGCAAAGCTCCTGCTTAGGTGCCTCCACGGCGGCAAGCATCAC
A R L A Y G R F E D E S T E V P P F V V -
TTGGCGACCAGCGAGAATGCCGACCAGTCACAACAGATTTGGATGCCGGACACATTCTTC
361
420
AACCGCTGGTCGCTCTTACGGCTGGTCAGTGTTGTCTAAACCTACGGCCTGTGTAAGAAG
L A T S E N A D Q S Q Q I W M P D T F F -
CAAAATGAAAAAGAGGCACGACGACATCTCATAGACAAGCCGAACGTGCTCATTCGAATT
421
480
GTTTTACTTTTTCTCCGTGCTGCTGTAGAGTATCTGTTCGGCTTGCACGAGTAAGCTTAA
Q N E K E A R R H L I D K P N V L I R I -
CACAAGGACGGCTCGATCCTTTACAGCGTTAGGTTATCTCTGGTGCTGTCCTGCCCCATG
481
540
GTGTTCCTGCCGAGCTAGGAAATGTCGCAATCCAATAGAGACCACGACAGGACGGGGTAC
H K D G S I L Y S V R L S L V L S C P M -
TCATTGGAGTTCTACCCGTTGGATCGACAGAACTGCCTTATCGATCTCGCATCATATGCG
541
600
AGTAACCTCAAGATGGGCAACCTAGCTGTCTTGACGGAATAGCTAGAGCGTAGTATACGC
S L E F Y P L D R Q N C L I D L A S Y A -
TACACGACGCAGGACATCAAGTACGAATGGAAGGAGCAGAATCCGGTCCAGCAGAAGGAC
601
660
ATGTGCTGCGTCCTGTAGTTCATGCTTACCTTCCTCGTCTTAGGCCAGGTCGTCTTCCTG
Y T T Q D I K Y E W K E Q N P V Q Q K D -
GGCTTACGTCAGTCATTGCCAAGTTTCGAATTGCAAGATGTCGTCACCAAGTACTGCACC
661
720
CCGAATGCAGTCAGTAACGGTTCAAAGCTTAACGTTCTACAGCAGTGGTTCATGACGTGG
G L R Q S L P S F E L Q D V V T K Y C T -
AGTAAAACCAATACCGGAGAATACAGTTGTGCTCGGGTCAAACTTCTCTTGCGAAGAGAG
721
780
TCATTTTGGTTATGGCCTCTTATGTCAACACGAGCCCAGTTTGAAGAGAACGCTTCTCTC
S K T N T G E Y S C A R V K L L L R R E -

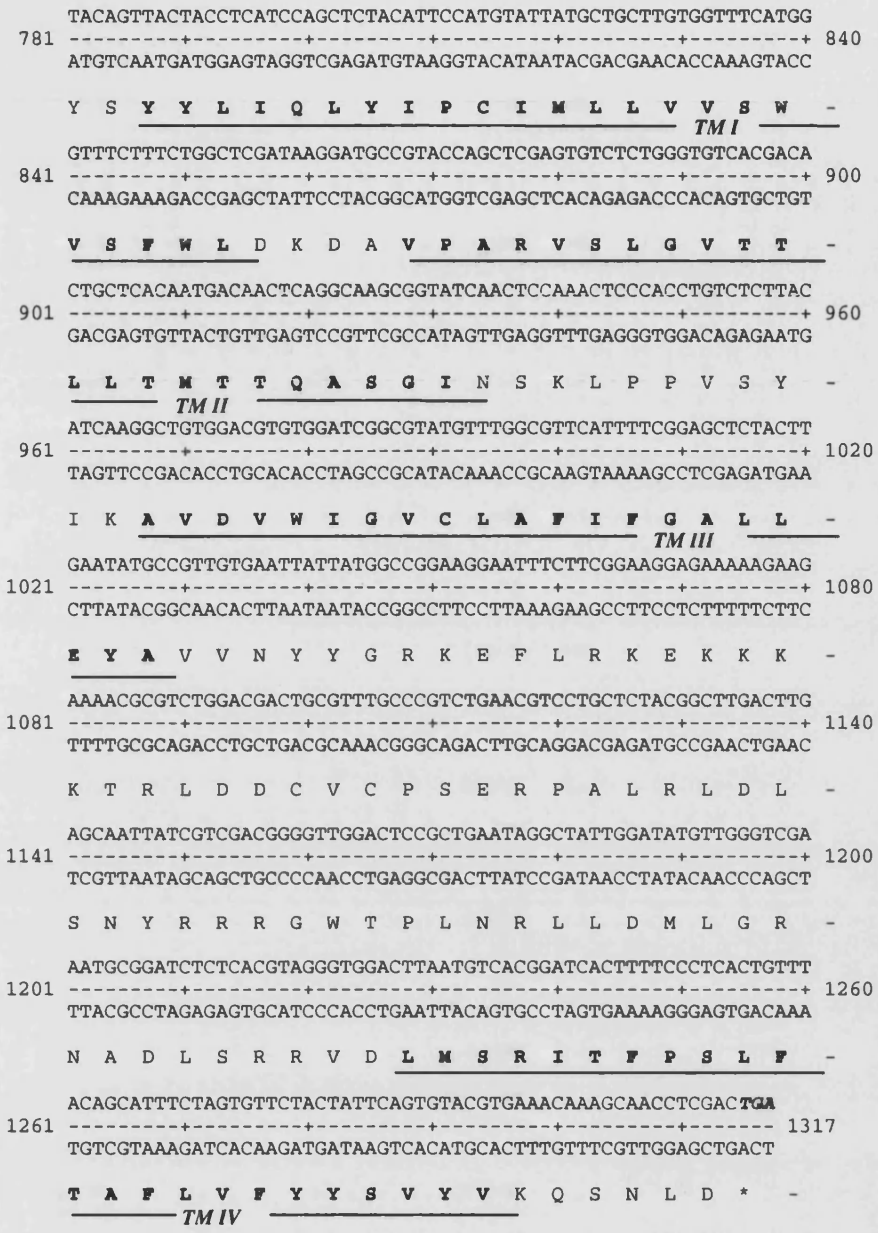


Figure 3.7 Nucleotide and predicted amino acid sequence of the *Hc-gbr-2b* clone. The sequence coding for the NH_2-terminal domain is shown in colour. The predicted start and stop codons are shown in bold italics. Region encoding the transmembrane domains are shown in bold. The sequence was submitted to EMBL Nucleotide Sequence Database and assigned the accession no. HCY14234.

Genus	Accession no.	Type of receptor subunit
C. elegans	U4113	Inhibitory amino acid receptor subunit; *Ce-gbr-2b* mRNA
C. elegans	U40573	Inhibitory amino acid receptor subunit; *Ce-gbr-2a* mRNA
Onchocerca volvulus	OVU59745	Glutamate-gated chloride channel (*GluCl X*); mRNA
C. elegans	U59743	Glutamate-gated chloride channel (*GluCl X*); precursor RNA
Dirofilaria immitis	U59744	Glutamate-gated chloride channel (*GluCl X*); mRNA
C. elegans	U14524	Avermectin-sensitive glutamate-gated chloride channel; *GluCl*α mRNA

Table 3.2 Results of the FASTA search in GenEMBL comparing the *Hc-gbr-2b* mRNA query sequence in the database. Sequence matches are listed in decreasing order of identity at the nucleotide level. *Hc-gbr-2a* mRNA gave a similar result.

```
    ATGCCGGACACATTCTTCCAAAATGAAAAAGAGGCACGACGACATCTCATAGACAAGCCG
    ATGCCGGACACATACTTCCAAAATGAAAAAGAGGCACGACGACATCTCATAGACAAGCCG

135 M  P  D  T  F/YF  Q  N  E  K  E  A  R  R  H  L  I  D  K  P   154


    AATGCGGATCTCTCACGTAGGGTGGACTTAATGTCACGGATCACTTTTCCCTCACTGTTT
    AATGCGTATCTCTCACGTAGGGTGGACTTAATGTCACGGATCACTTTTCCCTCACTGTTT

401 N  A D/Y L  S  R  R  V  D  L  M  S  R  I  T  F  P  S  L  F   420
```

Figure 3.8 Alignment of the *Hc-gbr-2b* cDNA sequence and the original, partial HG2/3 clone, showing differences (in bold) due to polymorphism. Amino acid positions correspond to those shown in Figure 3.6C.

Hc-GBR-2B	VPARV **S** LGVTTLLTMTTQ **A** SGIN
Ce-GBR-2B	VPARV **S** LGVTTLLTMTTQ **A** SGIN
Hc-GBR-2A	VPARV **T** LGVTTLLTMTTQ **S** SGIN
Ce-GBR-2A	VPARV **T** LGVTTLLTMTTQ **S** SGIN

Table 3.3 Comparison of the derived amino acid sequence in the TM II region of GBR-2A and 2B receptor subunits in *H. contortus* and *C. elegans*. Variable positions are shown in bold.

Hc-GBR-2B	L **M** S R I **T** F P **S** **L** **F** **T** **A** F **L** V **F** Y **Y** **S** V Y **V** - -
Ce-GBR-2B	L **M** S R L **T** F P **L** **T** **F** **F** **S** F **L** **I** **F** Y **Y** **V** **A** Y **V** - -
Hc-GBR-2A	- **V** S R L **V** F P **I** **G** **Y** **A** **C** F **N** V **L** Y **W** **A** V Y **L** - -
Ce-GBR-2A	- **V** S R L **V** F P **I** **G** **Y** **A** **C** F **N** V **L** Y **W** **A** V Y **L** - -

Table 3.4 Comparison of the derived amino acid sequence in the TM IV domains of GBR-2A and 2B receptor subunits in *H. contortus* and *C. elegans*. Variable positions are shown in bold.

3.2.2 Genomic PCR across the transmembrane/extracellular junction of Hc-GBR-2A and Hc-GBR-2B

Genomic PCR across the transmembrane/extracellular boundary of Hc-GBR-2A and 2B was performed in order to further investigate if the subunits were alternatively spliced products of the same gene. PCR was carried out such that the sequence of the amplification product included the region encoding the TM IV domain of the Hc-GBR-2A subunit. Genomic DNA was isolated from 2g of adult *H. contortus*, yielding 0.1mg DNA suspended in 1ml TE buffer (pH 7.6). 5µl was run on a 0.4% (w/v) agarose gel (Figure 3.9). 3µl genomic DNA was used in a touchdown-PCR amplification using Vent® DNA polymerase, sense primer 5HG2PR1 and antisense primer 3HG3PR1 designed to match regions across the transmembrane/extracellular boundary of the Hc-GBR-2A and 2B subunits respectively. The reaction was carried out with 3mM [Mg^{2+}] ion concentration (See Section 2.11.3). A 0.33Kbp band was amplified (Figure 3.10). DNA from the gel fragment was eluted (Section 2.10.2) and sequenced using primer 5HG2PR1 (Section 2.13.3D) (Figure 3.11). The sequence of genomic DNA matched exactly with the cDNA. The sequence across the transmembrane/extracellular boundary also corresponded with intron/exon splice sites, confirming that the *Hc-gbr-2a* gene was indeed spliced. The sequence of the second ORF encoding the second channel domain began 143 nucleotides downstream the termination codon of the Hc-GBR-2A subunit.

The sequence obtained was also compared with the genomic organisation across the transmembrane boundaries of the *gbr-2* gene in *C.elegans*. Genomic DNA was isolated from 2g of a developmentally mixed population of *C.elegans*, yielding 0.1mg (Figure 3.12). Touchdown PCR was carried out using primers Ceg2S1 and Ceg3AQ1 with 1µl genomic DNA and Vent® DNA polymerase. An approximately 460bp product was amplified (Figure 3.13) and sequenced (Figure 3.14). The sequence matched with that in the genEMBL database, corresponding to cosmid B0207 in the *C.elegans* genome sequencing project. It also matched exactly the cDNA sequence of *Ce-gbr-2a*. It differs from *Hc-gbr-2a* in that the second ORF encoding the second channel domain begins 120 nucleotides downstream from the termination codon of the GBR-2A subunit.

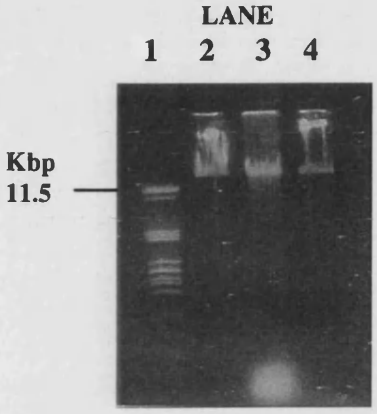


Figure 3.9 Genomic DNA isolated from adult *H. contortus* by the Herman Frischauf method. 0.4% (w/v) agarose gel in TBE with **Lane1**: λ/*PstI* DNA marker; **Lane2,4**: 0.7μg and 0.4μg λ DNA, **Lane3**: genomic DNA

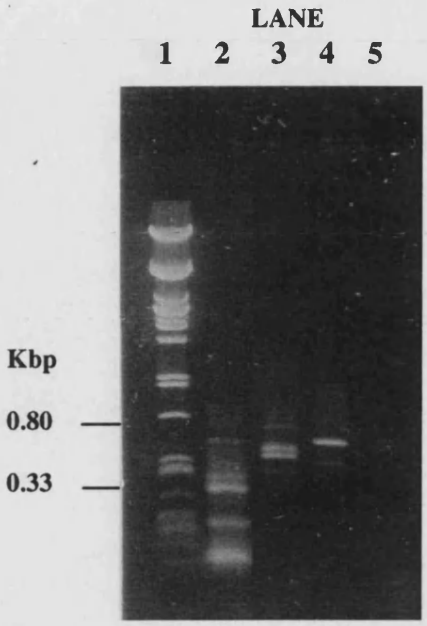


Figure 3.10 PCR amplification across the region encoding the transmembrane/extracellular boundaries of Hc-GBR-2A and Hc-GBR-2B. 1% (w/v) agarose gel in TBE with **Lane1**: λ/*PstI* DNA marker; **Lane2**: PCR product; **Lane3,4**: Single primer control PCR reactions with 5HG2PR1 or 3HG3PR1 alone; **Lane5**: No template control PCR reaction.

GGTGTTCCCTATTGGATATGCCTGTTTCAATGTGTTATATTGGGCTGTCTACTTGATGTGAagcatgga
V F P I G Y A C F N V L Y W A V Y L M *
TM IV
gcgtgctctactcgttcgtccgtccgtccgtccgtcctcattgtacctccgaactctcctcgtctcact

tttcttctctcaattcttcgcctcgttgctttccatctcacccagtgccttattagtcgtttatag

GGAGAATACAGTTGTGCTCGGGTCAAACTTCTCTTGCGAAGA***GAGTACAGTTACTACCTCATCC***
G E Y S C A R V K L L L R R E Y S Y Y L I
TM I

Figure 3.11 Sequence of PCR amplified product from *H. contortus* genomic DNA across the region encoding the transmembrane/extracellular boundary of Hc-GBR-2A (in blue) and Hc-GBR-2B (in black). The intron region is shown in lower case. PCR amplification was carried out using sense primer, 5HG2PR1 (in bold and blue) and antisense primer, 3HG3PR1 (primer sequence is complementary to the site shown in black in bold-italics).

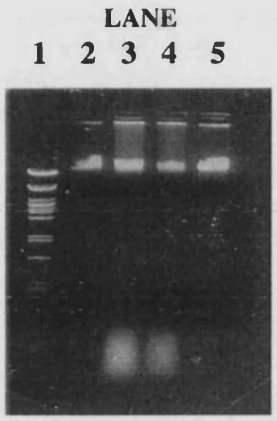


Figure 3.12 Genomic DNA isolated from a mixed population of *C.elegans* by the Herman Frischauf method. 0.4% (w/v) agarose gel in TBE with **Lane1**: λ/*PstI* DNA marker; **Lane2,5**: 0.2μg and 0.6μg λ DNA, **Lane2,3**: 5μl and 2μl genomic DNA.

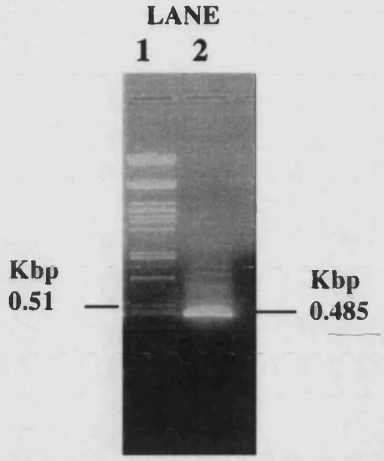


Figure 3.13 PCR amplification of genomic DNA across the region encoding the transmembrane boundaries of Ce-GBR-2A and Ce-GBR-2B. 1% (w/v) agarose gel in TBE with **Lane1**: λ/*PstI* DNA marker; **Lane2**: PCR product.

```
  1 AACGAATTGATGTTGTCTCCAGATTGGTGAGTTTTGTGTTTCGTATAATATTCTAAGGACTCTTCACGCAT  70
      R  I  D  V  V  S  R  L  ----------------------------------------------

 71 AGAAAGTTGTTTTAACAATTGAACGGCGTTGATTTACCAAAAGTTCTAATTGACCCTTTTCTGTCCTCCGC 140
    ------------------------------------------------------------------------

141 GGTGCCGTCAATAGCTTAGCGGAGCAACTAGTTGGTAGAGCGGGGGACTGTTCTTTTTTTTTGTTCATTTT 210
    ------------------------------------------------------------------------

211 TCAAATCAATTGTTTTTCAGGTCTTCCCAATCGGCTACGCGTGCTTCAACGGTACCTAGTTGATGTGAATG 280
    --------------------V  F  P  I  G  Y  A  C  F  N  --------------------

281 TTATATGTCTAATTCTTGAACTTTTTCAGTGCTTTATTGGGCCGTCTACCTGATGTGAATGCGTGTGCTAA 350
    --------------------------V  L  Y  W  A  V  Y  L  M  *  -------------

351 ACATTCCCTCATGTCTTCTATATATGTCTTCCTTTATGTGCCTTATCCCCCGGTTTACTTTGAATGTGTTT 420
    ------------------------------------------------------------------------

421 TCGAATGGACCAAATAATTTTTGTCGACGTATTTAGGTGAATACAGTTGTGCACGGGTCGTTTTGC 485
    ----------------------------------G  E  Y  S  C  A  R  V  V  L
```

Figure 3.14 Sequence of genomic PCR amplified product from *C. elegans* (Figure 3.15) across the region encoding for the transmembrane boundaries of Ce-GBR-2A and Ce-GBR-2B. Sense primer Ceg2S1 and antisense primer, Ceg3QA1 (primer sequence is complementary to the site) are shown in bold. Potential splice sites at intron-exon boundaries are in bold italics.

3.2.3 Sequence of *Hc-gbr-2a* and *Hc-gbr-2b* cDNA from ivermectin resistant *H. contortus*

Sequence of the *Hc-gbr-2* gene was also examined from IVR resistant isolates of *H. contortus* in an attempt to identify putative IVR resistance associated mutations. Total RNA was isolated from 1g of eggs from IVR resistant *H. contortus*. A yield of 0.8mg was suspended in 0.5ml of 0.5% (w/v) SDS (Figure 3.15). Poly[A]$^+$ RNA was purified from 200μg total RNA and then reverse transcribed to cDNA. This was used in PCR reactions carried out in triplicates. Although changes in the nucleotide sequence were observed, there were no relevant amino acid substitutions in either *Hc-gbr-2a* or *2b* cDNA. An amino acid change of a Thr residue (ACA) in place of a Ser residue (TCA) in the TM II region was observed in only one PCR amplification and not in two others, and was thus excluded as a PCR induced error (Figure 3.16).

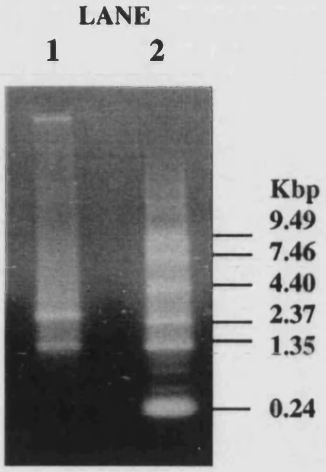


Figure 3.15 Total RNA isolated from eggs from ivermectin resistant *H. contortus* isolates. **Lane1**: total RNA; **Lane2**: 0.24-9.5Kb RNA ladder (1μg/μl)

GTACCAGCTCGAGTGTCTCTGGGTGTCACG**T/A**CACTGCTCACAATGACAACTCAGGCAAGCGGTATC
V P A R V S L G V T T/S L L T M T T Q A S G I

Figure 3.16 Nucleotide and amino acid changes observed in one of the PCR amplifications of *Hc-gbr-2b* cDNA from ivermectin resistant *H. contortus* isolates. Thr, TCA was replaced by Ser, ACA. The amino acid sequence is shown in bold italics.

3.2.4 Relative expression levels of *Hc-gbr-2a* and *Hc-gbr-2b* mRNAs

Initial attempts were made to obtain quantitative data on the relative expression levels of *Hc-gbr-2a* and *Hc-gbr-2b* mRNAs using Northern blots (Section 2.9.6). However, the low levels at which these mRNAs were expressed made it impossible to reliably detect them by Northern blotting. The RT-PCR approach was therefore made to analyse the relative levels of expression of the two mRNAs (Section 3.1.2).

0.5µl of the cDNA synthesised using eggs from IVR susceptible *H. contortus* was used in PCR amplifications (Section 3.2.1.1). Primers 5HG2PR3 and 3HG2PR1 were used for amplification of *Hc-gbr-2a* cDNA; primers 5HG3PR2 and 3HG3PR2 were used to amplify *Hc-gbr-2b* cDNA such that the amplified products were of comparable sizes. A control amplification of HCL9 cDNA encoding the L9 ribosomal protein in *H. contortus* was also carried out using primers 5HCL9 and 3TSA2 (Skinner, 1997). This was chosen as a control as there was no reported evidence of its binding to ivermectin or any related compounds. The amplifications were carried out simultaneously using the same cDNA pool but in separate reaction mixtures. PCR conditions were a 2min hot start at 94°C, followed by 46 cycles of 94°C, 15sec; 62°C, 30 sec; 72°C, 45sec at 1.5mM Mg^{2+}. 10µl of the reaction mix was removed every three cycles, starting from cycle 25. The products were run on a ethidium bromide stained 1.2% (w/v) agarose gel (Figure 3.17). Distinct amplification products were noted from cycle 41 for *Hc-gbr-2a* cDNA, but cycle 29 for *Hc-gbr-2b HCL9* cDNA. Thus compared to *Hc-gbr-2b* many more cycles of amplification were required for *gbr-2a* to produce a band of comparable intensity.

The ratio between *Hc-gbr-2a* and *2b* mRNA transcripts was also determined in eggs from IVR resistant *H. contortus* isolates. In order to ensure that the concentration of the cDNA used was similar to that from IVR susceptible *H. contortus*, the two samples were run on a silver-stained gel and compared by densitometry (Section 2.13.2) (Figure 3.18). The volume of cDNA to be used in PCR amplification was calculated accordingly. PCR primers and conditions were as described above. 10µl of the reaction mix was removed every five cycles, starting from cycle 25. Similar difference in expression levels between the two transcripts was observed. Amplification products were observed from cycle 40 for *Hc-gbr-2a* cDNA but only from cycle 30 for *Hc-gbr-2b* cDNA (Figure 3.19).

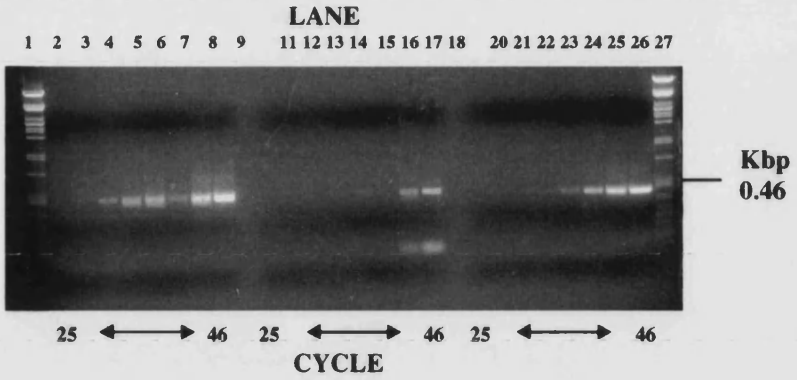


Figure 3.17 Semi quantitative PCR amplification of *Hc-gbr-2a* and *Hc-gbr-2b* cDNA from eggs from ivermectin susceptible *H. contortus*. 1.2% (w/v) agarose gel in TBE loaded with 10µl of each PCR sample removed after every 3 cycles from cycle 25. **Lane1, 28**: λ/*Pst* DNA marker; **Lane2-9**: *Hc-gbr-2b* cDNA; **Lane11-18**: *Hc-gbr-2a* cDNA; **Lane20-27**: *HCL9* ribosomal RNA control PCR amplification.

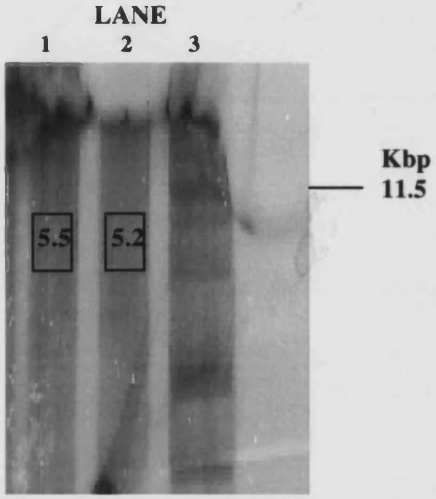


Figure 3.18 Silver stained gel (7.5 %) for a quantitative estimation of 5µl of cDNA from eggs from ivermectin susceptible (**Lane1**) and resistant (**Lane2**) *H. contortus*. **Lane3**: λ/*Pst* DNA marker. Densitometric values (in arbitrary units, O.D x mm^2) determined using the molecular analyst program 1.4.1 (Bio Rad) are shown for comparison.

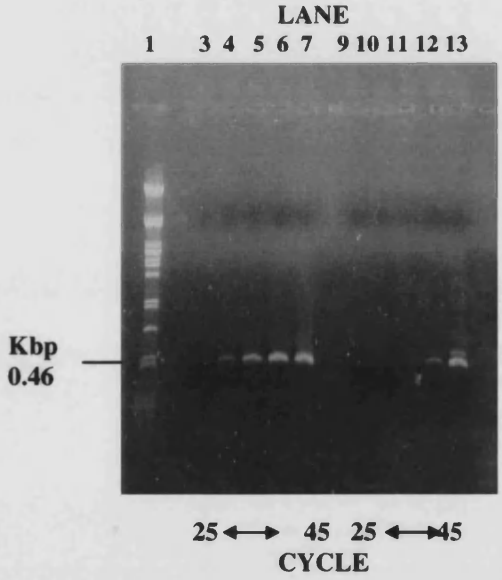


Figure 3.19 Semi quantitative PCR amplification of *Hc-gbr-2b* (**Lane2-6**) and *Hc-gbr-2a* (**Lane 9-13**) cDNA from eggs from ivermectin resistant *H. contortus* isolates on a 1.2% (w/v) agarose gel. 10µl of each PCR sample was removed after every 5 cycles from cycle 25. **Lane1**: λ/*Pst* DNA marker.

3.3 Discussion

cDNA cloning of the *gbr-2* gene from *H. contortus* confirms that it is alternatively spliced. It encodes two subunits, Hc-GBR-2A and Hc-GBR-2B which are members of the glutamate-gated chloride ion channel family of receptor subunits. The gene directs the synthesis of two mRNA species. This was confirmed by sequence determination of the full-length *Hc-gbr-2a* cDNA and cloning of the *Hc-gbr-2b* cDNA. The *Hc-gbr-2a* cDNA contains the channel encoding sequence of subunit Hc-GBR-2B within its 3′ untranslated region. Hc-GBR-2A and 2B share 82% identity at the amino acid level. Their derived amino acid sequences show the presence of a common NH_2-terminal extracellular domain and two independent sets of four hydrophobic transmembrane domains, TM I to TM IV with an intracellular loop between TM III and TM IV. This suggests the presence of common ligand-binding sites linked to slightly different chloride channels. The pattern of alternative splicing of the *Hc-gbr-2* gene and its genetic organisation suggests that the subunits encoded are orthologues of the GBR-2A and 2B subunits from *C. elegans*. Hc-GBR-2A and 2B share 88% and 83% amino acid identity with Ce-GBR-2A and 2B respectively. The TM domains are highly conserved. The TM II channel encoding domains are in particular identical except for residues in two positions- Thr_{295} and Ser_{308} in GBR-2A are substituted with Ser_{295} and Ala_{308} in GBR-2B. This is interesting as a single mutation of Ala→Ser was made in the position equivalent to Ala_{308} in the TM II domain of the $GABA_A$ receptor/chloride ion channel gene from cyclodiene susceptible *Drosophila melanogaster* strains. The amino acid residue was shown to be responsible for its association with resistance to dieldrin and picrotoxin (ffrench-Constant *et. al.*, 1993a; Edwards and Lees, 1997). Similar mutations of the Thr/Ser/Ala TM II residues in Hc-GBR-2A and 2B may prove to be useful. Mutations in the TM II residues can also be important as they line the ion conducting channel and determine several other functional properties such as ion conductance, ion selectivity and gating (Campos-Caro *et. al.*, 1996; Xu and Akabas, 1996).

Between the TM III and TM IV of both Hc-GBR-2A and 2B is an intracellular loop with potential phosphorylation sites (Swope *et. al.*, 1992). Hc-GBR-2B possesses consensus sites for phosphorylation by cAMP dependent protein kinase at Thr_{362} and two

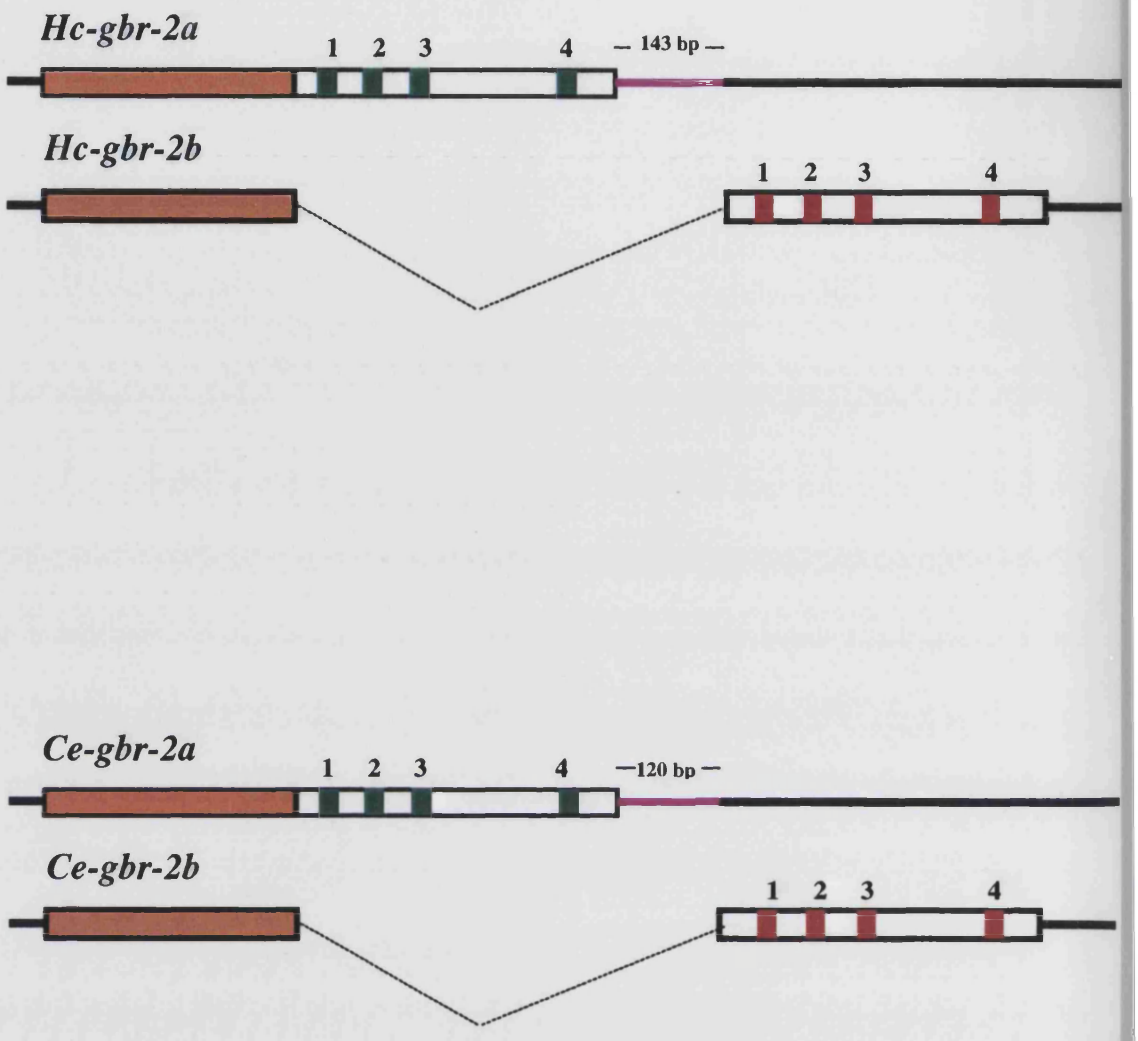


Figure 3.20 Schematic of the products GBR-2A and GBR-2B derived from the alternatively spliced *gbr-2* gene from *H. contortus* (above) and *C. elegans* (below). The common NH_2-terminal domains are shown in ▭, transmembrane domains are numbered 1-4 and the intron region shown in ▭.

protein kinase-C (PKC) sites at Ser_{371} and Ser_{405}. Ce-GBR-2B exhibits similar phosphorylation sites. Hc-GBR-2A and Ce-GBR-2A both on the other hand lack the corresponding cAMP dependent protein kinase sites. However Hc-GBR-2A has only one PKC phosphorylation site on Ser_{393}. It is possible that the varying number and position of the phosphorylation sites between GBR-2A and GBR-2B subunits is a means of subunit regulation.

The region across *Hc-gbr-2a* encoding for the TM IV domain and the 3′ UTR sequence encoding the channel domain of the Hc-GBR-2B subunit were analysed by genomic PCR. The transmembrane boundaries corresponded with intron splice sites. The sequence determining the second channel domain began 143 nucleotides after the termination codon of the Hc-GBR-2A subunit. A schematic of the structure is shown in Figure 3.20. Genomic PCR amplifications across *Ce-gbr-2* matched the cDNA sequence in the region encoding for the second channel domain which began 120 nucleotides downstream from the termination codon of Ce-GBR-2A (Figure 1.13A).

The *Hc-gbr-2* cDNA was amplified and sequenced from ivermectin resistant *H. contortus* isolates. IVR has been reported to act by opening chloride channels associated with GABA receptors on somatic musculature of parasitic nematodes, resulting in flaccid paralysis (Wang *et. al.*, 1980; Turner and Schaeffer, 1989). Activation of chloride channels was also observed in *Xenopus* oocytes injected with mRNA from *C. elegans* (Arena *et al.*, 1991). In addition, a single amino acid substitution in the susceptible strain of *Drosophila* mutant *Rdl* (resistant to dieldrin) was found to be associated with resistance (ffrench-Constant *et. al.*, 1993). *H. contortus* is also well documented for polymorphic variations within different populations. Kwa *et. al.* (1993) have for example, shown this by restriction fragment length polymorphism between benzimidazole sensitive and resistant populations of *H. contortus*. Most nucleotide polymorphisms require comparison with at least three independent amplifications, and at best in different populations (Hoekstra *et. al.*, 1997). In view of such reports, the *Hc-gbr-2* sequence was examined from IVR resistant isolates of *H. contortus* in three separate PCR amplifications. But no relavant amino acid substitutions were observed, thus ruling out any resistance associated mutations. However, recently IVR resistance in

H. contortus was shown to be mediated by multidrug resistance proteins called P glycoproteins. These were originally identified in mammalian tumor cells causing multidrug resistance against anti-cancer drugs (Gottesman and Pastan, 1993). The possibility of involvement of the P glycoprotein followed mainly from the mammalian evidence of its binding to and transport of ivermectin in normal tissues (Pouliot and Heureux, 1997). An alteration in the restriction pattern was observed in the genomic locus of P glycoprotein derived from IVR-selected strains of *H. contortus* when compared with unselected strains (Xu *et. al.*, 1998). This suggests that an alteration in the gene structure or transcription of the P glycoprotein may involve resistance to IVR or its analogs.

The relative expression levels of *Hc-gbr-2a* and *2b* mRNAs was examined in eggs from IVR susceptible and resistant isolates of *H. contortus* and found to exhibit a quantitative difference. *gbr-2a* mRNA was expressed at much lower levels than *gbr-2b*. This is interesting as the *gbr-2a* and *2b* mRNAs from *C. elegans* do not exhibit any gross variations in mRNA levels (See Figure 1.13B) (Laughton *et. al.*, 1997). Altered expression levels of the *Hc-gbr-2a* and *2b* cDNAs may be a means of regulation of the two products. Further analysis of the expression levels through the developmental stages of the parasite can therefore be important for a better understanding. In *C. elegans*, qualitative reverse transcriptase PCR through synchronous cultures through different developmental stages showed the presence of both *gbr-2a* and *2b* transcripts throughout its life cycle, with no large variations in mRNA levels (Laughton, *pers. comm.*).

In conclusion, the *gbr-2* gene is conserved in both *H. contortus* and *C. elegans* at the sequence level. The *Hc-gbr-2* gene is alternatively spliced like the *Ce-gbr-2* gene and encodes two receptor subunits, Hc-GBR-2A and Hc-GBR-2B. Sequence analysis shows that they are members of the glutamate-gated chloride ion channel family of receptor subunits. Hc-GBR-2A exhibits exhibits highest amino acid identity with Ce-GBR-2A and Hc-GBR-2B with Ce-GBR-2B. Sequence analysis of the *Hc-gbr-2* gene from ivermectin resistant isolates of *H. contortus* does not exhibit any variations at the amino acid level when compared with the sequence from IVR susceptible isolates. *Hc-gbr-2a* and *2b* cDNAs differ in their levels of expression unlike *Ce-gbr-2a* and *2b* which show similar levels of expression. Thus, the results suggest that although orthologues of the *C. elegans*

GBR-2A and 2B subunits occur in *H. contortus* , their expression levels may vary. The *Ce-gbr-2* gene has also been shown to map very close to the *avr-14* gene responsible for high-level synthetic resistance to ivermectin (Dent and Avery, 1998). Given the similarity in the genetic organization of the two species, it is possible that *Hc-gbr-2* also exhibits such features. However, this requires further examination.

(4) CONSTRUCTION AND SCREENING OF A cDNA LIBRARY FROM *ASCARIS SUUM*

4.1 Introduction

Glutamate-gated chloride (GluCl) ion channels are important targets for the widely used avermectin class of anthelminthics used to treat parasitic helminths and insect pests of man and animals. GluCl channels have been reported in invertebrates including nematodes (Cully *et. al.*, 1996), crustaceans, (Cleland and Selverston, 1995), insects (Horseman *et. al.*, 1988; Wafford and Sattelle, 1989), and molluscs (Magazanik *et. al.*, 1990; Quinlan *et. al.*, 1995). Among nematodes, avermectin-sensitive GluCl channels were first cloned from *C. elegans* (Cully *et. al.*, 1994). Among parasitic nematodes such as *A. suum* although extensive pharmacology of ligand-gated chloride ion channel family of receptors is available there is little sequence information to support it. Attempts were therefore made to obtain the sequence of a receptor subunit in this family from *A. suum*.

Two methods commonly used to clone and identify full-length cDNAs are library screening and PCR. The PCR approach was therefore made initially to amplify a partial receptor subunit using cDNA reverse transcribed from mRNA purified from *A. suum* muscle as template (Laughton, *pers. comm*). Use of degenerate primers DL11 and DL19 made to conserved motifs within the extracellular and first transmembrane (TM1) domain of aligned vertebrate and invertebrate $GABA_A$ receptor sequences resulted in a product ~450bp in size (See Appendix I). The PCR product was to be further examined for its sequence in the present study and used as probe to screen a cDNA library constructed from *A. suum*.

4.1.1 cDNA library

Construction of a cDNA library and its screening has the advantage of obtaining a full-length clone in a single step. A schematic of the steps involved is shown in Figure 4.1. *A. suum* heads were used in construction of a lambda-Uni Zap XR cDNA library. This involved generating stable double stranded DNA copies of mRNAs, followed by their

insertion into a self replicating lambda phage vector such as lambda ZAP (Huynh *et al.*, 1984; Short *et al.*, 1988). The Uni-ZAP XR vector system combines the high efficiency of lambda library construction and the convenience of a plasmid system with colour selection. The λ ZAP vector has signals for both initiation and termination of DNA synthesis from the f1 bacteriophage origin of replication. This allows for *in vivo* excision of the pBluescript phagemid, thus eliminating the need to subclone DNA inserts from the λ phage into a plasmid (Dotto *et al.*, 1984). The amplified library grown on XL1-Blue MRF′ cells result in blue plaques with nonrecombinants, and white plaques with recombinants. The special features of the vector are thus expected to significantly increase the rate at which clones are isolated and analysed.

4.1.2 Tissue

The cDNA library was constructed using *A. suum* heads dissected approximately 1.5cm from the anterior end. Previous reports on *A. suum* pharyngeal muscle recordings showed the presence of glutamate receptors which gated a chloride channel sensitive to the avermectin analogue, milbemycin D (Martin 1996). Application of glutamate results in a dose-dependent increase in chloride conductance. The pharyngeal muscle also responds to ivermectin which opens chloride channels on somatic muscle cells (Brownlee *et. al.*, 1997; Martin and Pennington, 1989). In view of such observations *A. suum* heads were used as the tissue source for construction of the cDNA library.

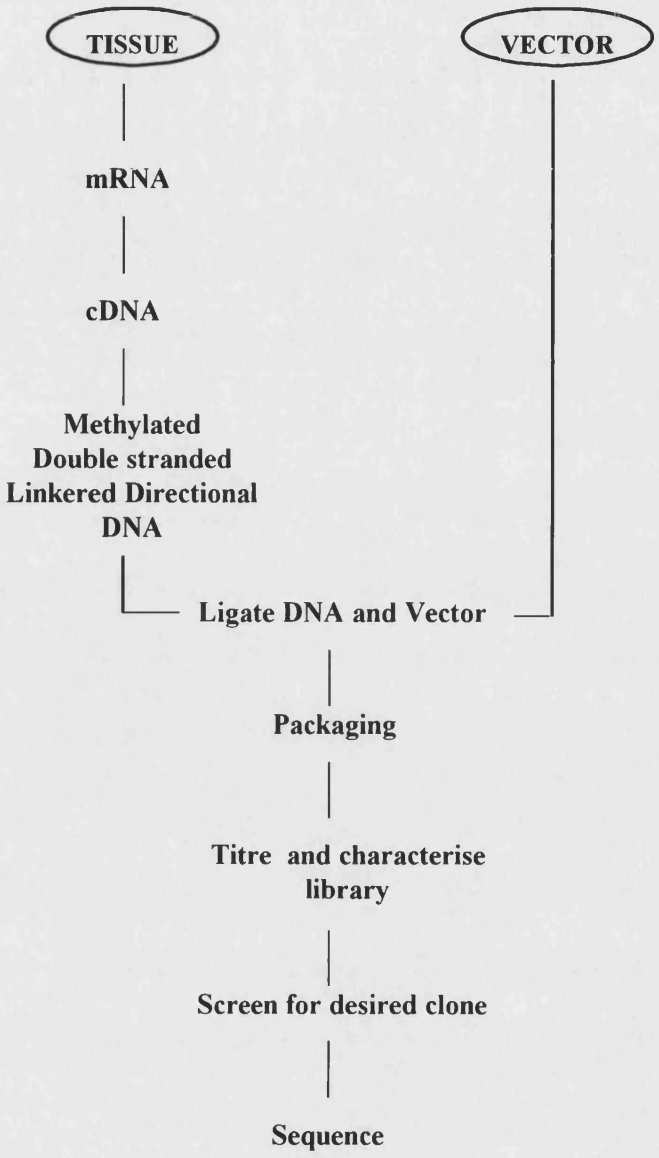


Figure 4.1: An outline of the steps involved in the construction of a cDNA library.

4.2 Results

4.2.1 Lambda ZAP II library construction

4.2.1.1 Template

The template for library construction was prepared as described in Section 2(C)2.15.1. The integrity of RNA was checked at each step as the quality and quantity of mRNA used are critical for representation of large messages in the cDNA library. 0.5µl of total RNA run on a 1% (w/v) RNase-free non-denaturing agarose gel showed two intact bands of sizes 28S and 18S (Figure 4.2). 400µg of total RNA (3mg/ml) was used to isolate poly-$[A]^+$ RNA and a 1µl aliquot of the sample run on the gel (Figure 4.2). Control reactions using test RNA provided in the kit were processed in parallel. 5µl and 1µl aliquots of products of first and second strand syntheses respectively were run on a 1.2% (w/v) agarose gel (Figure 4.3). A smear in the range of 4Kbp to 0.4Kbp was observed. Second strand synthesis of the control cDNA corresponded to a 1.8Kbp size product. cDNA termini were blunt ended, modified, and size fractionated (See Section 2.15). Concentration of size fractionated DNA was determined by ethidium bromide plate assay.

4.2.1.2 Ethidium bromide plate assay

0.8% (w/v) molten agarose in Tris-acetate buffer (pH 8.0) was cooled to 50°C and 10µl of ethidium bromide (10mg/ml) added. 10ml of agarose gel was poured per plate, allowed to set, and incubated at 37°C to dry. 0.5µl of seven serial dilutions of a standard DNA sample in 100mM EDTA (pH 8.0) (range of 200 to 10 ng/µl) were spotted on the surface. 0.5µl of *A. suum* cDNA was spotted adjacent to the standards within 10min of each other. The plate was left at room temperature for 15min to allow the spots to absorb, then inverted, and photographed using a UV lightbox. Two fractions of *A. suum* cDNA and test cDNA corresponded to approximately 50ng/µl (as estimated on the higher side) upon comparison with the standards (Figure 4.4). This was used for packaging into host strains

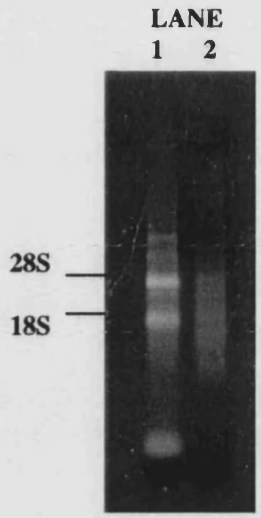


Figure 4.2 Total RNA and mRNA from *A. suum* heads run on a 1% (w/v) agarose gel in TBE. **Lane1**: 0.5µl total RNA (~1.5µg) showing ribosomal bands; **Lane2**: 1µl poly-$[A]^+$ RNA (~500ng) purified from the total RNA extracted.

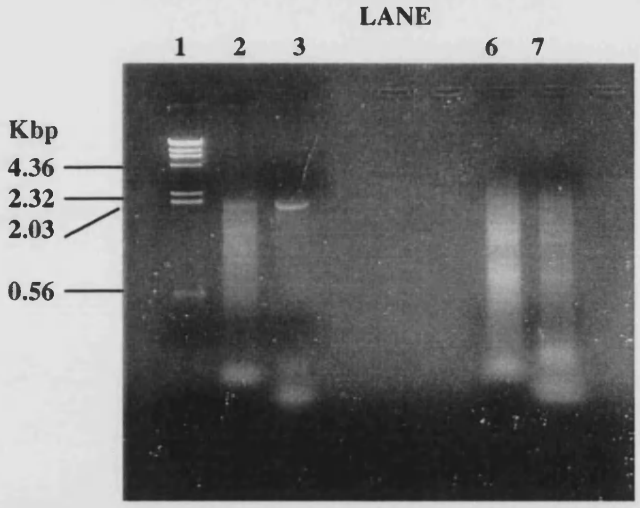


Figure 4.3 Products of first and second strand synthesis of *A.suum* mRNA run on a 1.2% (w/v) nondenaturing ethidium bromide stained agarose gel in TBE. **Lane1**: λ/*HindIII* DNA as molecular weight standards (0.3µg); 5µl and 1µl each of first and second strand syntheses products respectively using- **Lanes2, 3**: test RNA provided in the kit and **Lanes6, 7**: *A. suum* RNA. (Samples were prepared in a total volume of 10µl.

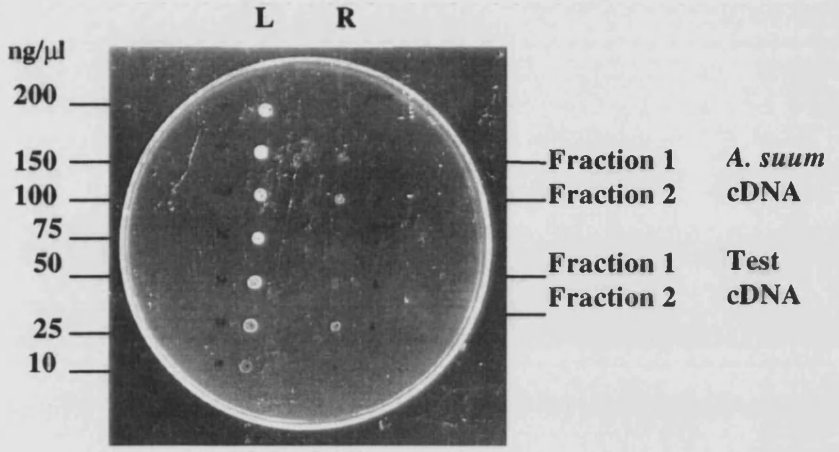


Figure 4.4 Ethidium bromide plate assay to estimate concentration of the size fractionated cDNA samples. **Left(L)**: Concentrations of ethidium bromide standards in ng/µl; **Right(R)**: 0.5µl of size fractionated samples 1 and 2 of *A. suum* cDNA and cDNA reverse transcribed from the test RNA provided in the kit.

1.1.1.1 DNA insert	*Vector used in ligation*	*Host bacterial strain*	*Dilution*	*~Plaque no* *White*	*Blue*
Packaging extract control, λ*c*I857 Sam7 wild type DNA	–	VCS257	10^{-4}	440	150
1.1.1.1.1 Kit test RNA control	Uni-ZAP XR	XL1-Blue MRF′	1 10^{-1}	260 120	10 2
PBR322 test insert ligation	Uni-ZAP XR	XL1-Blue MRF′	1 10^{-1}	540 80	
Ascaris head cDNA	Uni-ZAP XR	XL1-Blue MRF′	1 10^{-1}	900 100	16

Efficiency of the packaging reaction =

$$\frac{\text{No. of plaques x Dilution factor x Total packaging volume}}{\text{Total no. of μg packaged x No. of μl plated}} = \frac{440 \times 10^4 \times 1000}{0.2 \times 10} =$$

$$= 2.3 \times 10^{-9} \text{ pfu}$$

Phage titre of the constructed *Ascaris* cDNA library = 916 x 500 = 4.6 x 10^5 plaque forming units (pfu) / packaging reaction in a 500μl vol.

No. pfu of blue background plaques in the constructed *Ascaris* cDNA library / packaging reaction in a 500μl vol. = 16 x 500 = 8 x 10^3 pfu

This was in 1/5 the total vol. of the ligation mix.

∴ no. blue pfu / μg of arms = 8 x 10^3 = 4 x 10^4 pfu

Expected no. of blue background plaques is < 1 x 10^5 pfu/μg of arms

Table 4.1 Number of plaques noted after packaging *A. suum* cDNA and each of the controls provided in the kit into their respective host strains. 1μl out of a total 500μl volume was plated for each packaging reaction of the positive controls of a) packaging extract (λcI857 Sam7 wild type DNA); b) test RNA; c) test insert ligation (pBR322); and d) ligated *A. suum* cDNA. Efficiency of packaging was determined from the number of plaques obtained by packaging the wild type λ*c*I857 *Sam*7 lambda control DNA into the Gigapack III Gold Packaging Extract.

4.2.2 Library amplification

2µl (100ng) of each cDNA was used for ligation with 1µg of Uni-ZAP-XR vector. 1µl of the ligation mix was packaged into Gigapack III® gold Packaging extract using host strains XL1-Blue-MRF' and VCS257 (See Section 2.15.4 for genotypes of strains). Packaging reactions using 1µl (0.2µg) of wild type λ*c*I857 *Sam*7 DNA as positive control for the packaging extract was carried out in parallel. The number of plaques obtained for each packaging reaction were counted. Efficiency of the packaging reaction and phage titre of the *A. suum* cDNA library were determined (Table 4.1). The library had ~ 4.6 x 10^5 unique recombinants observed as clear plaques. Blue coloured background plaques were < 1 x 10^5 pfu/µg of arms of Uni-ZAP-XR vector. Since inserts were cloned directionally between *EcoRI-XbaI* restriction sites, the library was screened by restriction digests for cloned insert cDNA sizes. pBluescript DNA was rescued from five lambda plaques picked at random from the library. The SOLR strain was used for *in vivo* excision as it eliminated problems associated with helper phage co-infection, allowing efficient excision of the pBluescript phagemid from the Uni-ZAP XR vector. Restriction digests of DNA isolated from overnight cultures of excised phagemids were run on a 1% (w/v) agarose gel. Insert cDNA sizes were between 0.5Kbp and 1.2Kbp for three clones. One clone contained an insert of size 0.45Kbp and the other was undigested (Figure 4.5). The library was finally amplified in order to obtain a large an stable quantity of a high titre stock. The titre was determined as 8.8 x 10^9 pfu/ml, with a total volume of 120ml.

4.2.3 DNA Probe for library screening

4.2.3.1 Subcloning partial receptor subunit from *A. suum* and sequence analysis

A PCR product amplified using degenerate primers DL11 and DL19 (See Appendix I) was blunt ended and subcloned into *SmaI* digested pBluescript SK+ vector. Eleven resulting clones were checked by *XbaI-XhoI* restriction digests (Figure 4.6). Three clones showed presence of an insert, two of which were with inserts of the expected size of 450bp. All clones were manually sequenced by Sanger's chain termination method using pBluescript sense T3 and antisense M13 primers. Sequence of two clones, pASJ1.1 and pASJ1.8 had the four cysteine residues known to be conserved

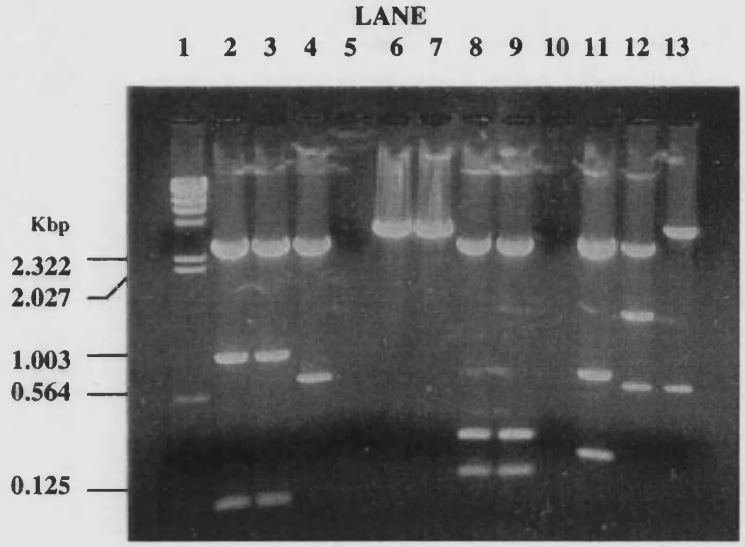


Figure 4.5 Restriction digests of *in vivo* excision clones to screen for the size of insert cDNAs in the library. 1% (w/v) nondenaturing ethidium bromide stained agarose gel in TBE showing products of digestion with enzymes *EcoRI* and *XhoI*. pBluescript DNA rescued from five Lambda plaques were picked at random from the library. **Lane1**: λ/*HindIII* molecular weight standards; **Lane2, 3**: Insert size 1.13Kb; **Lane 4**: Insert size 0.86Kb; **Lane 6,7**: undigested phagemid DNA; **Lane 8,9**: Insert size 0.46Kb; **Lane11**: Insert size 0.82Kb; **Lane12**: Control cDNA double digest; **Lane13**: Control cDNA single digest.

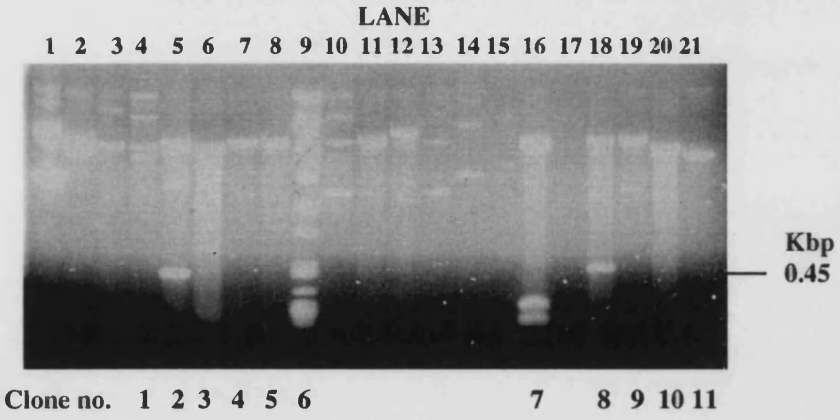


Figure 4.6 Restriction digests of plasmid DNA from recombinant clones of *Asg-2* in pBluescript SK^+ vector. 1% (w/v) agarose gel in TBE with **Lane2**: undigested pBluescript DNA; **Lane3**: single enzyme digested pBluescript DNA; **Lane10**: λ/*PstI* DNA marker; **Lanes4-9, 16-21**: *XbaI* / *XhoI* digested plasmid DNA of recombinant clones with lanes6,17 and 18 showing presence of insert. **Lanes10-12**: Single enzyme (*XbaI*) digests of plasmid DNA of clones 2, 6, 8 chosen in random; **Lanes 13-15**: undigested plasmid DNAof clones 2, 6, 8.

in the ligand-gated ion channel family of receptor subunits (Figure 4.7). Another striking feature confirming amplification of an authentic partial receptor subunit was the presence of transmembrane domain I. The sequence showed high amino acid identity with other partial receptor subunits from *C. elegans* and *H. contortus* (Table 4.2).

Wizard DNA™ miniprep of an overnight culture of pASJ1.1 was digested with enzymes *XhoI* and *XbaI* and run on a 1% (w/v) agarose gel. The 450bp size band corresponding to *Asg2* DNA was purified from the gel using Sephaglas™ (See Section 2.10.2) and used as a DNA probe. The probe was labeled as described in Section 2.9.6E and hybridized to plaque lifted membranes of the amplified library. Primary screening of ~10^6 plaque forming units (pfu) was carried out. Seven plaques which appeared to show a positive signal after primary screening were cored and secondary screening carried out (Section 2.15.7). However this did not result in the identification of any clone corresponding to the *Asg2* gene.

```
CCTGACACATTCTTCCAGAACGAAAAGGAGGCGAGGAGACACCTCATCGACAAGCCGAAT
 P  D  T  F  F  Q  N  E  K  E  A  R  R  H  L  I  D  K  P  N -

GTGTTGATTCGAATTCATCAGGACGGGCAAATATTGTATAGCGTTCGGTTATCATTGGTG
 V  L  I  R  I  H  Q  D  G  Q  I  L  Y  S  V  R  L  S  L  V -

CTGTCTTGTCCGATGTCGCTCGAATATTATCCGCTCGATCGACAGACATGTCTCATCGAT
 L  S  C  P  M  S  L  E  Y  Y  P  L  D  R  Q  T  C  L  I  D -

CTCGCTAGCTATGCATACACAACAGACGACATAAAGTACGAGTGGAAACTGACGAACCCA
 L  A  S  Y  A  Y  T  T  D  D  I  K  Y  E  W  K  L  T  N  P -

ATTCAGCAAAAGGAAGGTCTTCGGCAGAGTTTGCCTAGCTTCGAGTTGCAGGACGTGCTC
 I  Q  Q  K  E  G  L  R  Q  S  L  P  S  F  E  L  Q  D  V  L -

ACCGATTATTGCACCAGTAAGACGAACACAGGTGAATACAGCTGTGCCCGTGTCAAGCTA
 T  D  Y  C  T  S  K  T  N  T  G  E  Y  S  C  A  R  V  K  L -

CTGCTTCGTCGCGAATACAGTTATTATTTAATTCAGCTCTACATCCCGTGCATTATGCTC
 L  L  R  R  E  Y  S  Y  Y  L  I  Q  L  Y  I  P  C  I  M  L -

GTCGTCGTCTCTTGGGTCTCCTTCTGG
 V  V  V  S  W  V  S  F  W
```

Figure 4.7 Sequence of insert *Asg2* DNA amplified from *A. suum* and cloned into pBluescript. Sequences of inserts in both plasmids pASJ1.1 and 1.8 were the same. Sequence motif to which degenerate primers were designed for use in PCR amplification of the partial receptor subunit are shown in bold. The four cysteine residues are underlined.

Receptor type	Genus	Accession no.	% amino acid identity
Hc-GBR-2B	*Haemonchus contortus*	Y14234	92%
Ce-GBR-2B	*Caenorhabditis elegans*	U41113	91%
Ce-GBR-2A	*Caenorhabditis elegans*	U40573	86%
Hc-GBR-2A	*Haemonchus contortus*	Y14233	85%
Ce-Glu-Clα	*Caenorhabditis elegans*	U14524	69%
HG5 (Hc-GluClα like)	*Haemonchus contortus*	*Pers. comm.*	68%
Ce-Glu-Clβ	*Caenorhabditis elegans*	U14525	58%
HG4 (Hc-GluCl β like)	*Haemonchus contortus*	Y09796	57%
Rat glycine receptor α1	*Rattus rattus*	D00833	43%
GABA	*Lymnaea stagnalis*	X58638	27%

Table 4.2 Percent amino acid identity of partial receptor subunit Asg2 from *A. suum* with other inhibitory ligand gated ion channel receptor subunits. GluCl subtypes represent glutamate-gated chloride ion channel subunits. GBR-2A and 2B are the alternatively spliced products of the *gbr-2* gene.

4.3 Discussion

4.3.1 cDNA library construction of *A.suum.*

The Zap cDNA® synthesis kit (Strategene) was used to construct a cDNA library from *A.suum* heads. A critical parameter in library construction was the use of high quality RNA used to generate cDNA. This was confirmed by obtaining undegraded and DNA-free mRNA. The cDNA generated was successfully ligated into Uni-Zap XR vector and packaged into Gigapack III® Gold Packaging extract. Packaging was confirmed by plating on IPTG-X-gal NZY agar plates which resulted in a low background of blue plaques. *In vivo* excision of pBluescript phagemid from the Uni-Zap XR vector finally resulted in cDNA of the expected range of sizes. This suggested that the cDNA library was of high quality and could be used again when required. The presence of cDNAs over a range with fewer cDNAs of small size suggested that perhaps secondary structure of mRNA was not a major problem during first and second strand syntheses.

4.3.2 Library Screening

Extensive screening of the amplified cDNA library from ascarid heads was carried out using *Asg2*, a 450bp partial receptor subunit isolated from *A. suum* muscle as probe. *Asg2* shows high identity with the alternatively spliced *gbr-2* genes in *Haemonchus contortus* and *Caenorhabditis elegans* respectively which encode for GluCl ion channel receptor subunits. Screening of the library under stringent conditions did not result in the identification of any clone corresponding to the *Asg2* gene. One possible reason may be very low mRNA levels of the gene encoding for *Asg2* in the anterior head region of the organism when compared to the mid region. In view of the high sequence identity of the *Asg2* and *Hc-gbr-2* genes, this may partly be supported by the observations made by immunocytochemical localisation experiments of the *gbr-2* gene in *H. contortus*. No staining in the anterior end including the pharynx was noted, suggesting that the *gbr-2* gene was not expressed in those regions (See Chapter 6). It is possible that expression of the *Asg2* gene is similarly restricted to regions apart from the head and the pharynx. This could perhaps explain the difficulty in detection of possibly a GBR-2 like receptor subunit cDNA in ascarid heads.

Electrophysiological studies using *A. suum* muscle strips show the presence of GABAergic receptors (Martin, 1980; Martin *et al.*, 1991; Bascal *et. al.*, 1996); however, the neurones in *A. suum* head have only recently been characterised and shown to be amenable to intracellular recording techniques (Holden-Dye and Walker, 1994). Since there is only indirect evidence from electrophysiological data for the presence of GABA/glutamate receptor subunits in the *A. suum* pharynx, it is possible that the responses observed correspond to mRNA transcripts different from *Asg2*. Screening with a mixed probe of closely related sequences of receptor subunit cDNAs can therefore be an alternative to using a specific probe such as *Asg2* and increase the probability of obtaining a rare transcript.

Attempts to identify full length cDNAs for GABA/glutamate receptor subunits by construction and screening of a cDNA library in *A. suum* by other workers have also proved to be less successful (Stretton, *pers. comm.*), suggesting that the mRNAs for these messages may be rare. Screening of several *Lymnaean* nervous system cDNA libraries for example, failed to result in any positive clones as the transcript was extremely low in abundance. The RACE-PCR method was therefore used to isolate and clone $GABA_A$ receptor subunit cDNAs from the mollusc *Lymnaea stagnalis* (Harvey, 1991). This technique has also resulted in the successful isolation of several inhibitory amino acid receptor cDNAs from *C. elegans* and *H. contortus* (See Chapter 3). Isolation of the full-length receptor subunit cDNA, *Asg2* from *A. suum* by the RACE-PCR method may similarly be a sensitive method of direct amplification of an extremely rare mRNA.

(5) AMPLIFICATION OF RECEPTOR SUBUNIT AS-GBR-2 FROM *ASCARIS SUUM*

5.1 Introduction

The presence of Glutamate-gated chloride (GluCl) ion channels in *Ascaris suum* as observed from pharyngeal recordings makes it an obvious target for molecular cloning studies (Martin, 1996). Attempts were therefore made to clone and identify a GluCl receptor from *A. suum*. A partial receptor subunit cDNA, *Asg2*, was amplified by PCR with degenerate oligonucleotide primers. cDNA isolated from *A. suum* muscle strips was used as template (Laughton *pers. comm.*). The derived amino acid sequence of *Asg2* shows high amino acid identity with GBR-2A and GBR-2B receptor subunits from *C. elegans* and *Haemonchus contortus*. GBR-2A and 2B are subunits derived from a single alternatively spliced *gbr-2* gene. In trying to obtain the full length sequence of *Asg2* or identify any other related cDNA's, this was used as probe to screen a cDNA library constructed from *A. suum* (Chapter 4). However, no positive clones were identified possibly due to low *Asg2* transcripts. The alternate approach of RACE-PCR rapid amplification of cDNA ends-polymerase chain reaction was therefore made to obtain the full-length sequence of *Asg2* (Saiki *et al.*, 1988). This method has been successfully employed to obtain full-length sequences of rare transcripts of other receptor subunits from *H. contortus* and *C. elegans* (Laughton *et. al.*, 1994; Delany. N., Skinner, T., *pers. comm*; See also Chapter 3)

5.1.1 RACE-PCR

Thermal RACE, also known as single-sided or 'anchored' PCR, is a technique by which 3′ and 5′ ends of a cDNA can be obtained with knowledge of a small stretch of sequence within an internal region of the cDNA (Frohman *et al.*, 1988;). This follows by extension of cDNAs from ends of the messages, to specific primer sequences. The yield of the desired product depends largely on the stringency of the amplification reaction and the relative abundance of the specific transcript within the mRNA source.

Attempts were made to obtain the 3′ RACE-PCR amplification product of *Asg2* from cDNA reverse transcribed from *A. suum* muscle strips. RoRi [dT], a 'hybrid'

primer consisting of a 28 base oligonucleotide with a stretch of 17 dT residues was used during reverse transcription (Frohman and Martin, 1989). The oligo-[dT] stretch binds the poly (A) tail and allows for transcription. Gene specific oligonucleotide primers made to *Asg2* and primer RoRi were used on this cDNA for PCR. 5′ RACE-PCR was carried out using a gene specific antisense primer and SL1 (spliced-leader) primer. The SL1 primer sequence corresponds to a small, 22 nucleotide, non-polyadenylated SL RNA which is *trans*-spliced on to primary RNA transcripts of most nematode mRNAs (Nilsen, 1993). Approximately, 90% of mRNAs in *A. suum* have been reported to acquire SL1 (Nilsen *et. al.*, 1989). 3′ and 5′ RACE-PCR products of *Asg2* thus obtained by this strategy were expected to result in amplification of authentic products and enable isolation of a full-length cDNA. RACE-PCR amplification products of *Asg2* were also examined by Southern hybridization analysis. A labeled oligomer or gene fragment from a region specifically contained within the amplified fragment was used as probe.

In order to determine if *Asg2* was alternatively spliced in a fashion similar to the *gbr-2* gene, RACE-PCR reactions on cDNA were carried out using primers designed to match sequences on either sides of the potential splice site. Sizes of the PCR amplification products were expected to vary in the event of alternative splicing of the *Asg2* gene. PCR amplifications were also attempted on *A. suum* genomic DNA to confirm the same.

5.2 Results

5.2.1 Template

A. suum muscle was dissected from adult worms supplied in warm Locke solution. The tissue was frozen under liquid nitrogen until further use. Total RNA was isolated from 1gm of muscle tissue yielding 0.5mg. This was suspended in 0.5ml of 0.5% (w/v) SDS. 1µl of the sample was run on a non-denaturing RNase-free gel (Figure 5.1). Both the 28S and 18S ribosomal bands were clearly visible suggesting that the RNA was not degraded. Poly $[A]^+$ RNA was isolated from 200µg of total RNA and reverse transcribed to cDNA using primer RoRidT. cDNA synthesis was checked by labeling an aliquot of the reverse transcription mix with [α-^{32}P] dATP and running on an alkaline agarose gel. cDNA of size 3Kbp to 0.5Kbp was observed. The cDNA was used to carry out PCR reactions.

5.2.2 3′ RACE-PCR

5.2.2.1 Amplification of nematode paramyosin

3′ RACE-PCR was carried out on a Perkin Elmer Cetus DNA-Thermocycler with 1µl of *A. suum* muscle cDNA and *Taq* DNA polymerase (Promega). Sense primers ASG1, ASG2 or ASG3 designed to match regions within *Asg2* were used in conjunction with primer RoRi (See Table 5.1). This did not result in amplification of receptor subunit cDNAs. Use of primer ASG-4 under PCR conditions of a 5min hot start at 95°C, followed by 40 cycles of 95°C, 1min; 60°C, 1min; 72°C, 2min and a final extension at 72°C for 10min resulted in the amplification of a 1.2 Kbp sized product. This appeared as an intense band when run on a 1% (w/v) agarose gel run in TBE (Figure 5.2). As estimated from sequence comparison with other related receptor subunits, the 3′ RACE-PCR product of *Asg2* was slightly larger than the predicted size. However, to confirm if the amplified product was authentic, the 1.2Kbp DNA was gel purified and manually sequenced using ASG-4 (See Section 2.13.3C). A 150bp sequence was obtained from an initial sequencing run (Figure 5.3a) and used to search the genEMBL database with the TFASTA program (Figure 5.3b). The derived amino acid sequence showed 93% amino acid identity with nematode paramyosin from *Onchocerca volvulus* and *Dirofilaria immitis*; 88% amino acid identity was noted with paramyosin encoded for by the *β unc-15* gene in *C. elegans*. The sequence obtained was thus very likely *A. suum* paramyosin.

Nonspecific amplification was perhaps due to use of excess template or a considerable difference in melting temperatures of the primer pairs used. The reactions were therefore carried out again under modified conditions.

Primer	Sequence	Tm (°C)	Peptide Motif
ASG-1	5′ GCTACTGCTTCGTCGCGAATACAG 3′	74	LLLREY
ASG-2	5′ ATTCAGCTCTACATCCCGTGC 3′	64	IQLYIPC
ASG-3	5′ TTGCACCAGTAAGACGAACAC 3′	64	CTSKTN
ASG-4	5′ TTATGCTCGTCGTCGTCTC 3′	58	MLVVV
ASG-5	5′ AACCCAATTCAGCAAAAGGAAGG 3′	64	NPIQQKE
5AS1	5′ AACACATTCGGCTTGTCGATG 3′	62	IDKPNV
5AS2	5′ CCACTCGTACTTTATGTC 3′	52	DIKYEW
5AS3	5′ AGCTGTATTCACCTGTGTTCG 3′	62	NTGEYS
5AS4	5′ GTACGACGAATGGTGGAACC 3′	62	VPPFVV
SL1	5′ GGTTTAATTACCCAAGTTTGAG 3′	60	
SL1b	5′ GGTTTAATTACCCAAGTTTG 3′	54	
RoRi	5′ GACTACGTTAGCATCTAGAATTCTCGAG 3′	80	
RoRidT	5′ GACTACGTTAGCATCTAGAATTCTCGAG $[T]_{17}$ 3′		

Table 5.1 Sense and antisense primers used in PCR amplification of *As-gbr-2* cDNA from *A. suum*

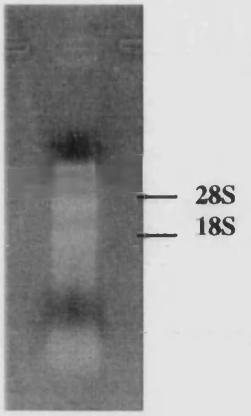


Figure 5.1 Total RNA isolated from *A. suum* muscle run on a 1% (w/v) agarose gel in TBE.

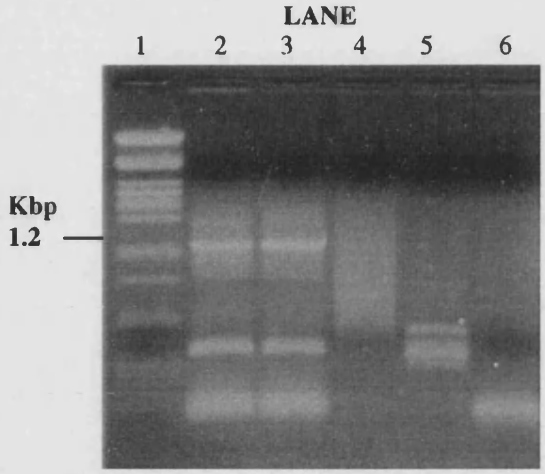


Figure 5.2 Amplification of *Ascaris* paramyosin from *A. suum* muscle cDNA. 1% (w/v) agarose gel in TBE with **Lane1**: λ*PstI* marker; **Lane2,3**: PCR amplification product; **Lane4**: single primer control with RoRi; **Lane5**: single primer control with ASG-4; **Lane6**: no template control.

```
   CTCGACTCAGTGCGCACAGCTCTCGACGAGGAATCCACCGCTCGTACTGAGGCCGAGCAC
 1 ---------+---------+---------+---------+---------+---------+  60
   L  D  S  V  R  T  A  L  D  E  E  S  T  A  R  T  E  A  E  H

   AAGCTTGCACTAGCCAACACCGAAATCACCCAGTGGAAGAGTAAATTCGACGCTGAAGTG
61 ---------+---------+---------+---------+---------+---------+ 120
   K  L  A  L  A  N  T  E  I  T  Q  W  K  S  K  F  D  A  E  V

   CACTGCACCATGAAGAGGTGGAAGACT
121 ---------+---------+------- 147
   H  C  T  M  K  R  W  K  T
```

Figure 5.3A Sequence of *Ascaris* paramyosin amplified during 3′ RACE-PCR reaction using *A. suum* muscle cDNA. Sequence of only a small region was determined. An exact match with primer ASG-4 was observed

```
O.v EEARRRLEDAERERSQLQAQLHQVQLELDSVRTALDEESAARAEAEHKLALANTEITQWKSK

A.s --ARRRLE------------------LDSVRTALDEESTARTEAEHKLALANTEITQWKSK

O.v FDAEVALHHEEVEDLRKKML

A.s FDAEVALHHEEVED------
```

Figure 5.3B Comparison of a part of the amino acid sequence of paramyosin mRNA from *Ascaris suum* (*A.s*) with *Onchocerca volvulus* (*O.v*). Primer ASG-4 used in PCR amplification shows an exact match in one of its reading frames with the *O.v* sequence, explaining the reason for non-specific amplification.

5.2.2.2 Amplification of 3′ RACE-PCR product of *Asg2*

1µl of a one in ten dilution of the cDNA template was used in conjunction with sense primer ASG-5 and antisense primer RoRi with the High Fidelity Expand™ PCR System. The cycling conditions were a hot start of 2min at 94°C, followed by 35 cycles of 94°C, 15sec; 64°C, 30sec; 72°C, 45sec and a final extension at 72°C for 7min. The reaction mix was run on a 1.5% (w/v) agarose gel run in TBE (Figure 5.4a). The gel was Southern blotted and hybridized to the internal oligonucleotide primer ASG-4. A positive signal was noted for a 0.8 Kbp band (Figure 5.4b). DNA was eluted from the band cut out from the gel using the Sephaglas kit and blunt ended (Section 2.12.3). This was subcloned into pBluescript vector DNA processed in a similar fashion. DNA from nine resulting clones were checked by *XbaI/XhoI* restriction enzyme digests (Figure 5.5). Two clones, pASJ2.1 and pASJ2.2 with the right size of insert were manually sequenced in both directions using T3 sense primer and M13 antisense primer.

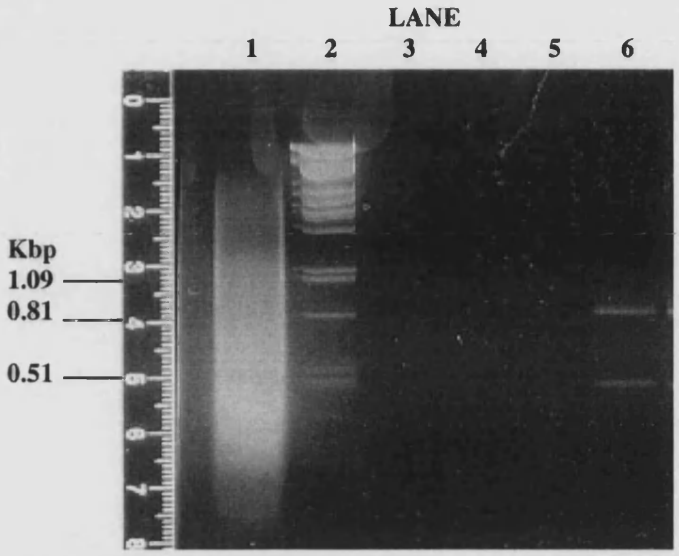


Figure 5.4A 3′ RACE-PCR amplification of *Asg2* from *A. suum* muscle cDNA. 1.2% (w/v) agarose gel in TBE with **Lane1**: Salmon sperm DNA; **Lane2**: λ/*Pst* DNA marker; **Lane3-5**: no template and single primer controls; **Lane6**: 3′ RACE-PCR amplification.

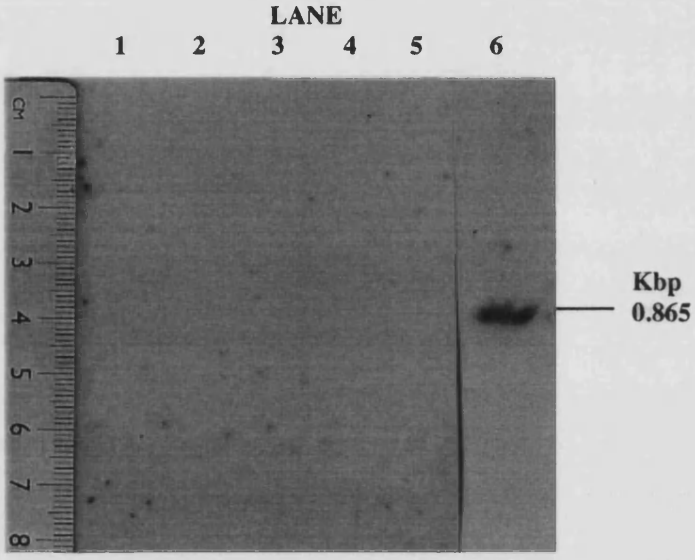


Figure 5.4B Southern blot analysis of 3′ RACE-PCR amplification products of *Asg2*. Agarose gel in 5.4a was hybridized to oligonucleotide primer ASG-3. Primer sequence corresponded to a region internal to the sense primer used in PCR. **Lane1-5**: Controls; **Lane6**: 3′ RACE-PCR product showing a positive signal.

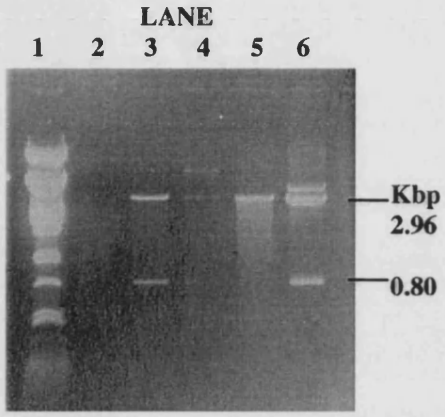
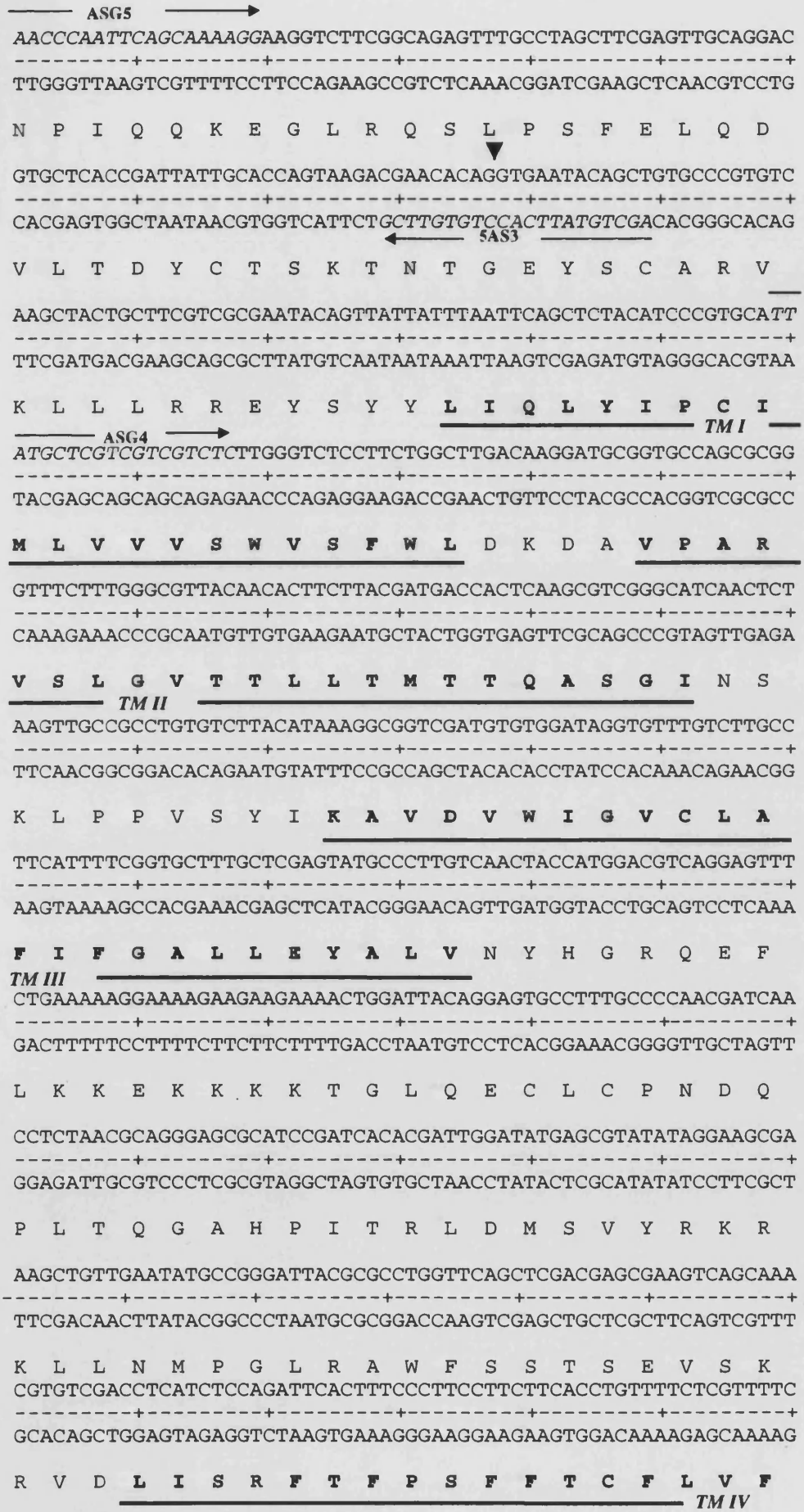


Figure 5.5 Restriction digests of plasmid DNA from clones of the 3′ RACE-PCR product of *Asg2* in pBluescript. **Lane1**: λ/*Pst* DNA marker; **Lane3-6**: *XbaI/HindIII* digested plasmid DNA.

5.2.2.3 Sequence Analysis

The sequence of the 0.8 Kbp 3′ RACE-PCR product of *Asg2* was clearly related to GluCl subunits (Figure 5.6). The derived amino acid sequence of the cDNA showed presence of all four transmembrane domains, TM I-TM IV, and an intracellular loop between TM III and TM IV. Sequences of *Asg2* and the 3′ RACE PCR product were merged into one single reading frame and searched for matches in genEMBL database using the TFASTA programme. High amino acid identity was once again observed with glutamate-gated chloride ion channel receptor subunits, GBR-2A and GBR-2B from *C. elegans* and *H. contortus* derived from the alternatively spliced *gbr-2* gene. A high 84% amino acid identity was observed with Hc-GBR-2B. This suggests that the sequence from *A. suum* also belongs to the *gbr-2* gene which encodes for the As-GBR-2 receptor subunit. The TM II domain of As-GBR-2 is identical to the GBR-2B subunits from *H. contortus* and *C. elegans* showing the presence of conserved Ser and Ala residues (See Table 5.2). The TM IV domain exhibits 74% and 70% identity with the TM IV domains of Hc-GBR-2B and Ce-GBR-2B respectively and only 56% identity with the 2A subunits (Table 5.3). Further sequence examination shows the presence of sites for phosphorylation by cAMP and cGMP dependent protein kinase at Thr 149, and by Casein kinase II at Thr 170 and Ser 193. However As-GBR-2 lacks a site for phosphorylation by protein kinase-C (PKC). Hc-GBR-2B in contrast bears two sites for PKC phosphorylation at Ser 371 and Ser 405 sites (See Chapter 3).

However, there is no evidence from the 3′ RACE-PCR to suggest the presence of multiple *As-gbr-2* mRNAs. This was confirmed by carrying out the PCR amplification using primer ASG-5, designed upstream to the potential splice site (shown by an arrow in Figure 5.6). In the event of alternative splicing of the *As-gbr-2* mRNA similar to the *gbr-2* genes in *C. elegans* and *H. contortus*, two 3′ RACE-PCR products corresponding to GluCl receptor subunits from a single PCR amplification would be obtained. However, this was not observed. A schematic representation of the PCR strategy is shown in Figure 5.7.


ASG5
AACCCAATTCAGCAAAAGGAAGGTCTTCGGCAGAGTTTGCCTAGCTTCGAGTTGCAGGAC
TTGGGTTAAGTCGTTTTCCTTCCAGAAGCCGTCTCAAACGGATCGAAGCTCAACGTCCTG
N P I Q Q K E G L R Q S L P S F E L Q D
GTGCTCACCGATTATTGCACCAGTAAGACGAACACAGGTGAATACAGCTGTGCCCGTGTC
CACGAGTGGCTAATAACGTGGTCATTCTGCTTGTGTCCACTTATGTCGACACGGGCACAG
5AS3
V L T D Y C T S K T N T G E Y S C A R V
AAGCTACTGCTTCGTCGCGAATACAGTTATTATTTAATTCAGCTCTACATCCCGTGCATT
TTCGATGACGAAGCAGCGCTTATGTCAATAATAAATTAAGTCGAGATGTAGGGCACGTAA
K L L L R R E Y S Y Y L I Q L Y I P C I
TM I
ASG4
ATGCTCGTCGTCGTCTCTTGGGTCTCCTTCTGGCTTGACAAGGATGCGGTGCCAGCGCGG
TACGAGCAGCAGCAGAGAACCCAGAGGAAGACCGAACTGTTCCTACGCCACGGTCGCGCC
M L V V V S W V S F W L D K D A V P A R
GTTTCTTTGGGCGTTACAACACTTCTTACGATGACCACTCAAGCGTCGGGCATCAACTCT
CAAAGAAACCCGCAATGTTGTGAAGAATGCTACTGGTGAGTTCGCAGCCCGTAGTTGAGA
V S L G V T T L L T M T T Q A S G I N S
TM II
AAGTTGCCGCCTGTGTCTTACATAAAGGCGGTCGATGTGTGGATAGGTGTTTGTCTTGCC
TTCAACGGCGGACACAGAATGTATTTCCGCCAGCTACACACCTATCCACAAACAGAACGG
K L P P V S Y I K A V D V W I G V C L A
TTCATTTTCGGTGCTTTGCTCGAGTATGCCCTTGTCAACTACCATGGACGTCAGGAGTTT
AAGTAAAAGCCACGAAACGAGCTCATACGGGAACAGTTGATGGTACCTGCAGTCCTCAAA
F I F G A L L E Y A L V N Y H G R Q E F
TM III
CTGAAAAAGGAAAAGAAGAAGAAAACTGGATTACAGGAGTGCCTTTGCCCCAACGATCAA
GACTTTTTCCTTTTCTTCTTCTTTTGACCTAATGTCCTCACGGAAACGGGGTTGCTAGTT
L K K E K K K K T G L Q E C L C P N D Q
CCTCTAACGCAGGGAGCGCATCCGATCACACGATTGGATATGAGCGTATATAGGAAGCGA
GGAGATTGCGTCCCTCGCGTAGGCTAGTGTGCTAACCTATACTCGCATATATCCTTCGCT
P L T Q G A H P I T R L D M S V Y R K R
AAGCTGTTGAATATGCCGGGATTACGCGCCTGGTTCAGCTCGACGAGCGAAGTCAGCAAA
TTCGACAACTTATACGGCCCTAATGCGCGGACCAAGTCGAGCTGCTCGCTTCAGTCGTTT
K L L N M P G L R A W F S S T S E V S K
CGTGTCGACCTCATCTCCAGATTCACTTTCCCTTCCTTCTTCACCTGTTTTCTCGTTTTC
GCACAGCTGGAGTAGAGGTCTAAGTGAAAGGGAAGGAAGAAGTGGACAAAAGAGCAAAAG
R V D L I S R F T F P S F F T C F L V F
TM IV

```
    TATTACGTGACATACGTGAAGTGAACGAAGAGGTGAACGAGGCGAGACTATGTTGTATAG
721 ---------+---------+---------+---------+---------+---------+ 780
    ATAATGCACTGTATGCACTTCACT

    Y  Y  V  T  Y  V  K  *

    AAGCACGCCACGATCCTATTGTTGCGTTGTTTTGCAATTAATTAACTGATTGTTAAAnTT
781 ---------+---------+---------+---------+---------+---------+ 840

    GTnAACCGCTAAAAAAAAAAAAAAA
841 ---------+---------+----- 865
```

Figure 5.6 Sequence of clone pASJ2 of the 3′ RACE-PCR product of the partial receptor subunit cDNA, *Asg2*, from *A. suum* muscle. The sequence corresponds to the *As-gbr-2* gene. Region to which sense primers ASG-4 and ASG-5 were designed to are shown. Transmembrane domains are underlined. The potential splice acceptor site is shown with an arrow (▼).

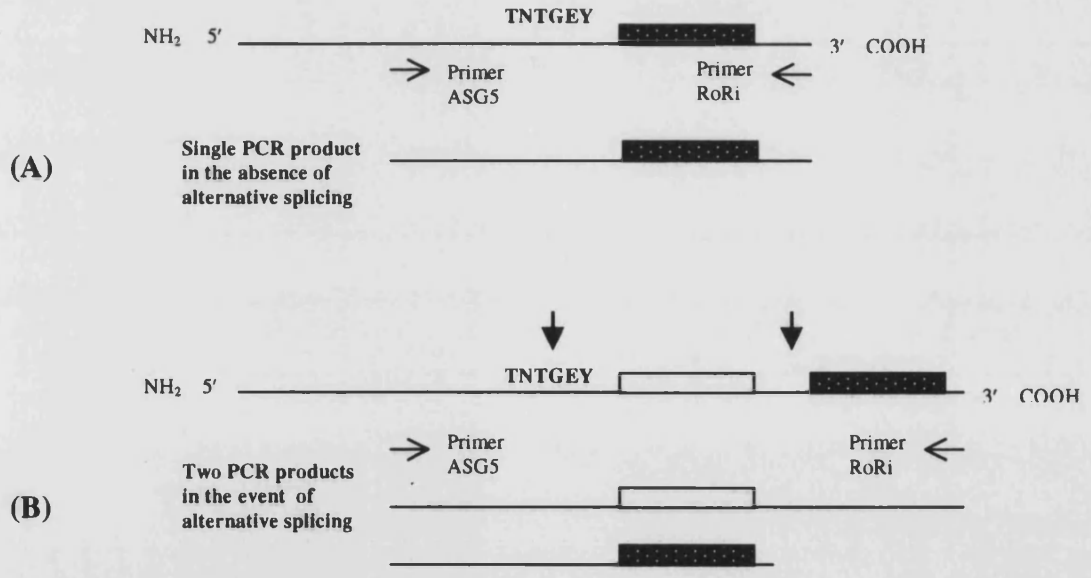


Figure 5.7 An outline of the PCR strategy to determine if the *As-gbr-2* gene is alternatively spliced. The potential splice site and the derived amino acid sequence in that region is shown with by a bold arrow. The number of possible PCR amplification products in **(A)** the absence of an alternative splicing and **(B)** in the event of alternative splicing are shown. Putative transmembrane domains are shown by ▭ and ▬.

Hc-GBR-2B	VPARV **S** LGVTTLLTMTTQ **A** SGIN
Ce-GBR-2B	VPARV **S** LGVTTLLTMTTQ **A** SGIN
As-GBR-2	VPARV **S** LGVTTLLTMTTQ **A** SGIN
Hc-GBR-2A	VPARV **T** LGVTTLLTMTTQ **S** SGIN
Ce-GBR-2A	VPARV **T** LGVTTLLTMTTQ **S** SGIN

Table 5.2 Comparison of the derived amino acid sequence in the TM II region of As-GBR-2 with other GBR-2A and 2B subunits. Variable positions are shown in bold.

Hc-GBR-2B	**L M S R** I **T F P** S L **F T** A **F L V F Y Y** S **V Y V** - -
Ce-GBR-2B	**L M S R L T F P** L T **F** F S **F L** I **F Y Y V** A **Y V** - -
As-GBR-2	**L** I **S R** F **T F P** S F **F T C F L V F Y Y V** T **Y V** - -
Hc-GBR-2A	- **V S R L V F P I G Y A C F N V L Y W A V Y L** - -
Ce-GBR-2A	- **V S R L V F P I G Y A C F N V** L **Y W A V Y L** - -

Table 5.3 Comparison of the derived amino acid sequence in the TM IV domains of GBR-2A and 2B receptor subunits with As-GBR-2. Amino acids common between the subunits are shown in bold.

5.2.3 5′ RACE-PCR

5.2.3.1 Amplification of 5′ RACE-PCR product of *Asg2*

5′ RACE PCR of *Asg2* was carried out using *A. suum* muscle cDNA as template and the High Fidelity Expand™ PCR System on a Perkin Elmer DNA Thermocycler. Sense primer SL1 and gene specific antisense primer 5AS3, or 5AS2 internal to 5AS3, designed to match complementary regions within *Asg2* were used. PCR cycling conditions were a hot start for 2min at 94°C, followed by 35 cycles of 94°C, 15sec; 50°C, 30 sec; 72°C, 1min. A final extension at 72°C for 7min was carried out. The reaction mix was run on a 1% (w/v) agarose gel in TBE. Several amplification products were noted. (Figure 5.8a). The gel was Southern blotted and hybridized to primer 5AS1, internal to both 5AS2 and 5AS3 (Figure 5.8b). DNA from bands of sizes 0.44 Kbp and 0.59 Kbp showing positive signals in PCR amplifications with primers 5AS2 and 5AS3 respectively were eluted. Their sequence was determined on the automated sequencer.

5.2.3.2 Sequence Analysis

The 5′ RACE-PCR product of *Asg2* resulted in the amplification of a GluCl related subunit (Figure 5.9). However, the 0.59 Kbp size cDNA amplified using primer 5AS3 was truncated at the 5′ end in its sequence, lacking the start codon and signal peptide. Sequence of the smaller 0.37 Kbp size cDNA amplified in the same PCR reaction also showing a positive signal upon hybridization to the internal primer made to *Asg2*, was also determined. Interestingly, this matched a smaller truncated 5′ RACE-PCR amplified receptor cDNA with SL1 at the 5′ end (shown with an arrow in Figure 5.9).

In an attempt to obtain product of larger size, PCR conditions were varied by increasing extension times, and altering annealing temperatures and primer pairs. As the quality of the reverse transcription reaction was important in obtaining full length cDNA's, PCR amplifications were carried out with fresh preparations of cDNA. The cDNA was reverse transcribed using RoRi [dT] primer or PoPi [dT] primer. PCR amplifications were also carried with an antisense primer, 5AS4, closer to the 5′ end. However, truncated products were obtained in each instance. To rule out mispriming and

obtain full length 5′ RACE PCR products, a slightly modified SL1b primer was designed. This matched the SL1 primer exactly in sequence but was shortened at the 3′ end by two bases. An overall sequence match with the spliced leader sequence was thus maintained, but with a decreased primer specificity on the 3′ end. PCR amplification products each time were Southern blotted and analysed. The spliced leader sequence was found at the 5′ end in each amplification and all products were truncated in their sequence.

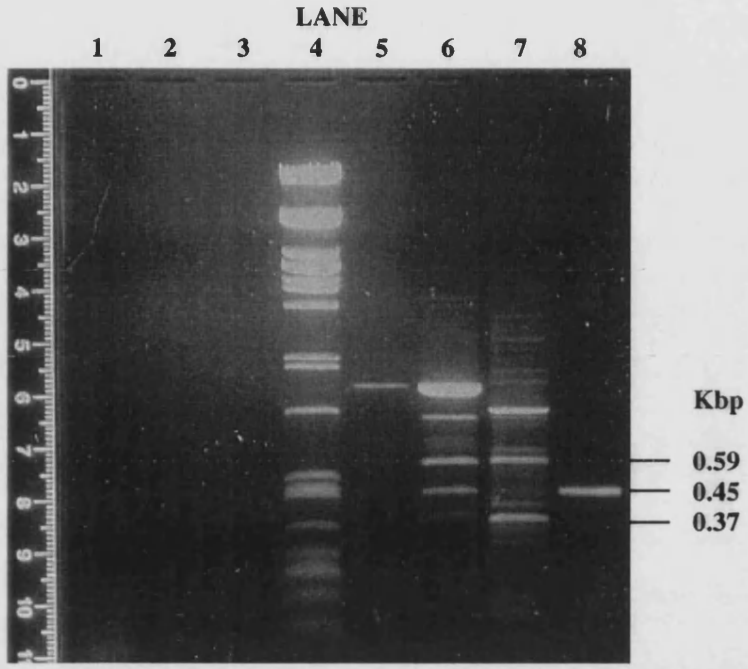


Figure 5.8A 5′ RACE-PCR amplification products of *Asg2*. 1% (w/v) agarose gel in TBE with PCR amplification products. **Lanes1,2,3**: No template and single primer controls; **Lane4**: λ/*Pst* DNA marker; **Lane5**: PCR using primers SL1 and 5AS2 at 30 cycles amplification; **Lane6**: PCR using primers SL1 and 5AS2 at 35 cycles amplification; **Lane7**: PCR using primers SL1 and 5AS3; **Lane8**: PCR of *Asg2*.

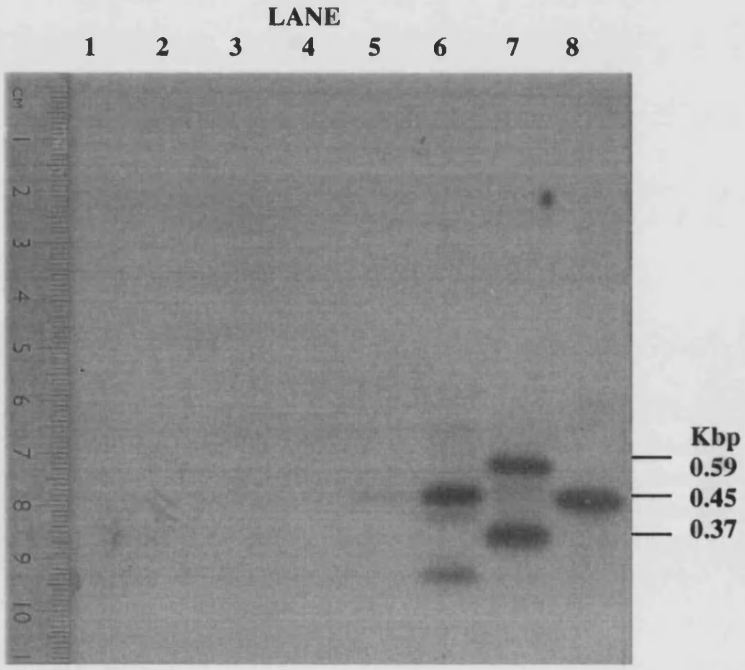
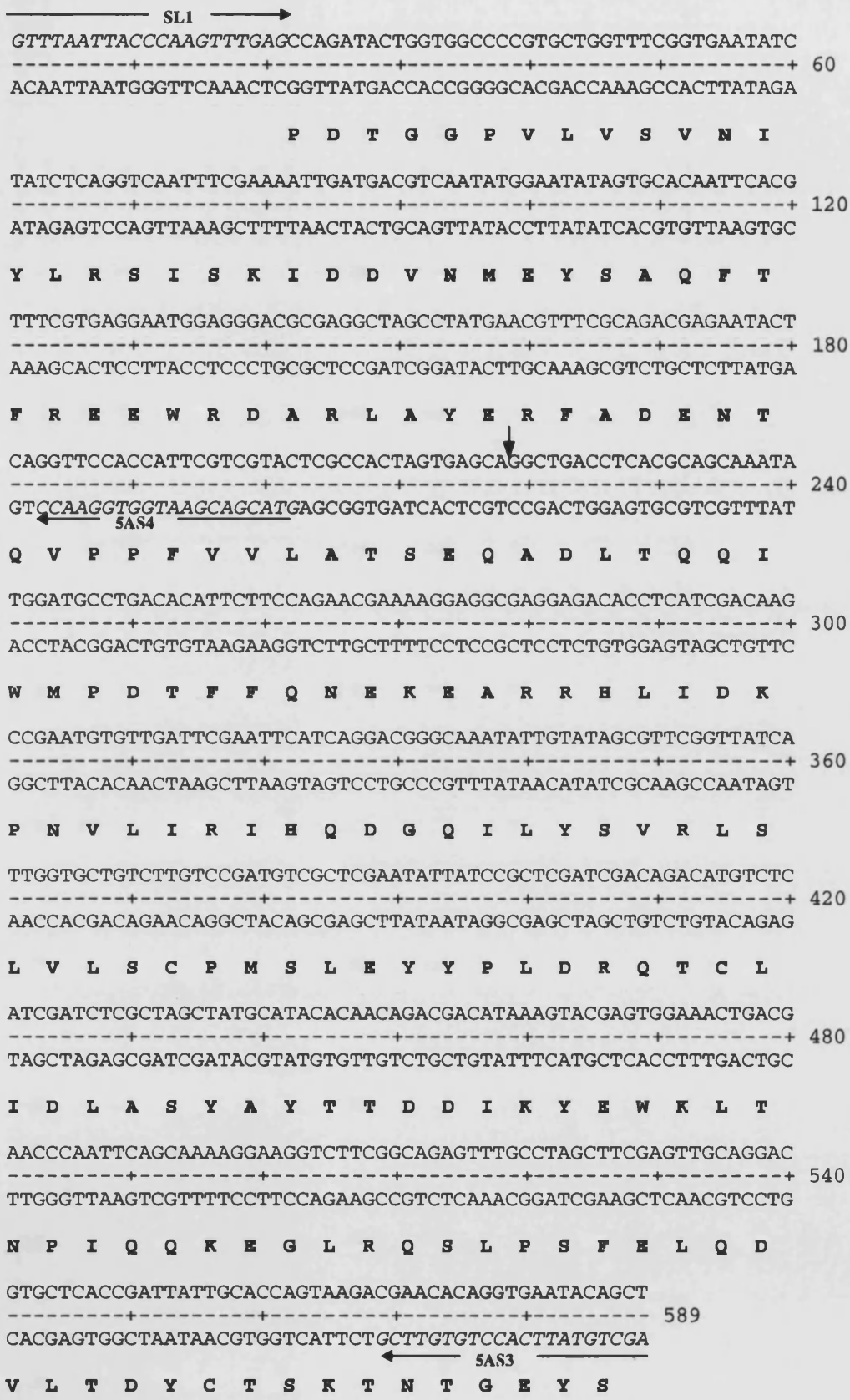


Figure 5.8B Southern blot of 5′ RACE-PCR products hybridized to the internal oligonucleotide primer, 5AS1 used as probe. 1% (w/v) agarose gel in TBE showing positive signals in lanes 6-9 after hybridization to the probe.

Figure 5.9 Sequence of the 5′ RACE-PCR amplified product of the partial receptor subunit cDNA, *Asg2*, from *A. suum* muscle. The sequence corresponds to the *As-gbr-2* gene. Sense primer SL1 and antisense primers 5AS3 and 5AS4 used in PCR amplifications are shown. The sequence of the smaller sized 0.37 Kbp PCR product amplified with primers SL1 and 5AS4 begins at the site shown by an arrow (↓).


SL1
5AS4
5AS3

5.2.4 Genomic PCR

An alternate approach to obtaining the 5′ end of the *As-gbr-2* cDNA was made by inverse PCR (Ochman *et. al.*, 1988; Triglia *et. al.*, 1988) (Section 2.11.4). Genomic DNA was isolated from 0.5g of adult *A. suum* by the Herman Frischauf (1987) method and suspended in 1ml of TE buffer (Section 2.8A). 5µl of the sample was run on a 0.4% (w/v) agarose gel in TBE and the yield estimated as ~10ng/µl. Spectrophotometric determination of the concentration was difficult due to contamination wiht muscle glycogen. Isolation of genomic DNA was therefore attempted by the CTAB method (Section 2.8B) reported to remove unwanted carbohydrate compounds (Damjana and Komel, 1994). However, the yield was only slightly improved to 30ng/µl by this method.

The isolation of genomic DNA was again attempted from 1gm adult *A. suum* using an improvised method of a shortened proteinase K step and the introduction of a potassium acetate step following it, as described in Section 2.8 (Adlouni *et. al.*, 1995). The DNA sample was finally dissolved in 100µl TE buffer (pH 7.6). 5µl of this genomic DNA was run on a 0.4% (w/v) agarose gel in TBE (Figure 5.10). This resulted in a much cleaner preparation with maximum yield of 50ng/µl when compared to all three different procedures carried out. However, the total amount of genomic DNA obtained was not sufficient to carry out restriction digests and Southern hybridization analysis using a probe made to *As-gbr-2*. At least 15µg of genomic DNA was required in order to determine the relevant sizes of enzyme digested genomic DNA hybridizing to the probe. Inverse PCR was therefore attempted using genomic DNA obtained from Pharmacia, Upjohn, U.S.A. 5µg genomic DNA was digested with restriction enzymes *EcoRI*, *NheI* or *NsiI* each. Restriction sites for the enzymes chosen were close to the 5′ end of *Asg2*. Enzyme digests were carried out at 37°C overnight and aliquots run on a 0.8% (w/v) agarose gel which was Southern blotted. These showed up as smears on the gel and not as specific bands as expected. Inverse PCR was carried out using 20µg genomic DNA as described in Section 2.11.4. However, this did not result in the amplification of any PCR products (Figure 5.11).

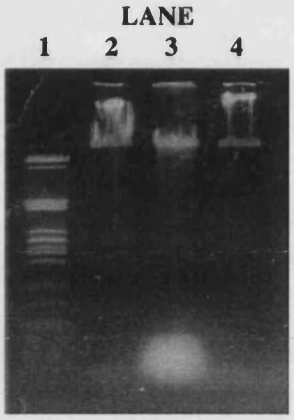


Figure 5.10 Genomic DNA isolated from adult *A. suum*. An improvised proteinase K method was used for the isolation procedure. **Lane1**: λ/*PstI* DNA marker; **Lane2,4**: Lambda DNA of concentrations 1.5μg and 0.75μg respectively; **Lane3**: 10μl of genomic DNA.

Figure 5.11 Inverse PCR on genomic DNA isolated from adult *A. suum*. **Lane1**: λ/*PstI* DNA marker; **Lane2,3**: Inverse PCR of *EcoRI* digested and circularised genomic DNA

5.3 Discussion

5.3.1 *As-gbr-2* gene

As-GBR-2 is clearly a member of the family of glutamate-gated chloride (GluCl) ion channel receptor subunits (See Appendix III). It is closely related to GBR-2A and GBR-2B subunits from *Caenorhabditis elegans* (Ce) and *Haemonchus contortus* (Hc) (Figure 5.12). The derived amino acid sequence of *As-gbr-2* shows highest amino acid identity of 82% and 84% with Ce-GBR-2B and Hc-GBR-2B subunits respectively. Sequence of the As-GBR-2 subunit in the TM II domain is identical to the GBR-2B subunits. Two residues Ser and Ala are conserved in this region (Table 5.2). These residues are substituted with amino acids Thr and Ser respectively in the TM II region of the GBR-2A subunits. Amino acid residues in these positions may be critical in determining the ion channel properties. Sequence alignment of the TM IV domains also shows that As-GBR-2 is closely related to the GBR-2B type of subunits (Table 5.3).

Although sequence data points to a greater relatedness of As-GBR-2 to the 2B subunits, there is no evidence of alternative splicing of *As-gbr-2* as observed for the *Ce-gbr-2* and *Hc-gbr-2* genes. This comes from Southern hybridization analysis of 3′ RACE-PCR products, amplified using the gene specific sense primer, ASG-4. The primer was specifically designed to match the *Asg2* sequence upstream from the potential splice site (shown by an arrow in Figure 5.6). In the event of splicing, two PCR products of sizes 0.86 Kbp and 0.76 Kbp were expected to show a positive signal upon hybridization to the probe. However, positive signal was noted only for the 0.8 Kbp size amplification product, suggesting that the *As-gbr-2* gene may not be alternatively spliced as noted for the *gbr-2* genes in *C. elegans* and *H. contortus*.

```
                                    ↓
           1                                                    50
Hc-GBR-2B  MRNSVPLATR IGPMLALICT VSTIMSAVEA KRKLKEQEII QRILNNYDWR
Ce-GBR-2B  .....MWHYR LTTILLIIS. ...IIHSIRA KRKLKEQEII QRILKDYDWR
As-GBR-2   .......... .......... .......... .......... ..........

           51    *                                             100
Hc-GBR-2B  VRPRGLNASW PDTGGPVLVT VNIYLRSISK IDDVNMEYSA HFTFREEWVD
Ce-GBR-2B  VRPRGMNATW PDTGGPVLVT VNIYLRSISK IDDVNMEYSA QFTFREEWTD
As-GBR-2   .......... .DTGGPVLVS VNIYLRSISK IDDVNMEYSA QFTFREEWRD

           101                                                  150
Hc-GBR-2B  ARLAYGRFED ES.TEVPPFV VLATSENADQ SQQIWMPDTF FQNEKEARRH
Ce-GBR-2B  QRLAYERYEE SGDTEVPPFV VLATSENADQ SQQIWMPDTF FQNEKEARRH
As-GBR-2   ARLAYERFAD E.NTQVPPFV VLATSEQADL TQQIWMPDTF FQNEKEARRH

           151                                                  200
Hc-GBR-2B  LIDKPNVLIR IHKDGSILYS VRLSLVLSCP MSLEFYPLDR QNCLIDLASY
Ce-GBR-2B  LIDKPNVLIR IHKNGQILYS VRLSLVLSCP MSLEFYPLDR QNCLIDLASY
As-GBR-2   LIDKPNVLIR IHQDGQILYS VRLSLVLSCP MSLEYYPLDR QTCLIDLASY

           201                                                  250
Hc-GBR-2B  AYTTQDIKYE WKEQNPVQQK DGLRQSLPSF ELQDVVTKYC TSKTNTGEYS
Ce-GBR-2B  AYTTQDIKYE WKEKKPIQQK DGLRQSLPSF ELQDVVTDYC TSLTNTGEYS
As-GBR-2   AYTTDDIKYE WKLTNPIQQK EGLRQSLPSF ELQDVLTDYC TSKTNTGEYS

           251                                                  300
Hc-GBR-2B  CARVKLLLRR EYSYYLIQLY IPCIMLLVVS WVSFWLDKDA VPARVSLGVT
Ce-GBR-2B  CARVVLRLRR EYSYYLIQLY IPCIMLVVVS WVSFWLDKDA VPARVSLGVT
As-GBR-2   CARVKLLLRR EYSYYLIQLY IPCIMLVVVS WVSFWLDKDA VPARVSLGVT

           301                                                  350
Hc-GBR-2B  TLLTMTTQAS GINSKLPPVS YIKAVDVWIG VCLAFIFGAL LEYAVVNYYG
Ce-GBR-2B  TLLTMTTQAS GINTKLPPVS YIKAVDVWIG VCLAFIFGAL LEYAVVNYYG
As-GBR-2   TLLTMTTQAS GINSKLPPVS YIKAVDVWIG VCLAFIFGAL LEYALVNYHG

           351                                                  400
Hc-GBR-2B  RKEFLRKEKK KKTRLDDCVC PSE....... RPALRLDLSN YRRRGWTP.L
Ce-GBR-2B  RKEFLRKEKK KKTRIDDCVC PSD....... RPPLRLDLSA YRSVKRLPII
As-GBR-2   RQEFLKKEKK KKTGLQECLC PNDQPLTQGA HPITRLDMSV YRKRKLLN.M

           401                                                447
Hc-GBR-2B  NRLLDMLGRN ADLSRRVDLM SRITFPSLFT AFLVFYYSVY VKQSNLD
Ce-GBR-2B  KRISEILSTN IDISRRVDLM SRLTFPLTFF SFLIFYYVAY VKQSRD.
As-GBR-2   PGLRAWFSST SEVSKRVDLI SRFTFPSFFT CFLVFYYVTY VK.....
```

Figure 5.12 Sequence alignment of the As-GBR-2 glutamate-gated chloride ion channel receptor subunit from *A. suum* with GBR-2 subunits from *C. elegans* (Ce) and *H. contortus* (Hc). Protein sequences were analysed with the GCG Pileup program. The signal peptide cleavage site is shown with an arrow, the N-linked glycosylation site is indicated with an asterisk, cysteine residues are underlined. Putative transmembrane domains are shown in bold.

5.3.2 RACE-PCR

In contrast to a complete 3′ RACE-PCR product of *Asg2*, a full-length 5′ RACE-PCR product was not obtained. Several attempts were made to amplify larger size products. Variations were made at the critical step of reverse transcription (RT) using a different oligo dT primer. The reaction was also carried out at a slightly elevated temperature of 42°C as cDNA synthesis at higher temperatures diminish the amount of secondary structure encountered in GC-rich regions of the mRNA. However, these changes did not alter obtaining truncated products. One way of maximizing cDNA extension can be by using a primer for the RT reaction close to the 5′ end of the region of the known sequence, combined with the use of a heat stable reverse transcriptase enzyme at elevated temperatures. This could improve the efficiency of cDNA extension by avoiding shorter specific cDNAs from becoming suitable templates for amplification during the RT reaction.

5′ RACE-PCR was also carried out by varying the cycle conditions and altering PCR primer pairs. Use of an antisense primer to match the *As-gbr-2* sequence closer to the 5′ end gave the same result. The SL1 primer was noted in the sequence of each PCR amplification product. In order to rule out mispriming of SL1 to a region downstream the authentic 5′ end of the *As-gbr-2* cDNA, a modified SL1 primer shortened by two nucleotides was used in PCR. However, this did not improve the result. More recent evidence in embryo extracts of *Ascaris* also suggests that *trans* splicing of SL1 occurs even with extensive alterations in its sequence or length (Davis, 1996; Maroney *et. al.*, 1991). It is possible that the observation of truncated products with SL1 in several PCR amplifications is due to the complex splicing machinery events occurring in *A. suum*, rather than a problem with the PCR conditions used. *Ascaris* exhibits extensive splicing (Komunuicki *pers. comm.*), with a large percentage acquiring the spliced leader sequence by *trans* splicing (Nilsen, 1993). By definition, *trans*-splicing involves the joining of two exons from two separately transcribed RNAs. The two substrates are brought together, at least in part, by a network of extensive base pairing interactions involving the SL RNA and other proteins of the splicing machinery (Nilsen, 1995). An alteration in this mechanism could very well alter the way in which SL1 *trans* splices. From nucleotide sequence comparisons of the 5′ RACE-PCR product of *As-gbr-2* at the site of truncation, with potential splice sites of other related receptor subunits, the possibility of *trans*

splicing of SL1 to an internal acceptor site (functional usually in *cis*) cannot be ruled out. Blumenthal and coworkers (1991) have shown *in vivo* in *C. elegans* and *in vitro* in *Ascaris lumbricoides* that a normal *cis*-splice acceptor site could function as a *trans* splice-acceptor. It is possible that the number of *As-gbr-2* mRNAs receiving SL1 at the *trans* splice acceptor site are very few compared to mRNAs that are favored to receive SL1 in the *cis* splice acceptor sites. If true, it may serve as a means of regulatory control of the number of functional copies of the *As-gbr-2* mRNA. This could in part explain the reason for the repeated preferential amplification of truncated 5′ RACE PCR products of *As-gbr-2* mRNAs *vis a vis* full length copies.

The use of a degenerate 5′ end primer in conjunction with a gene specific antisense primer may be a means of obtaining the full length sequence of the *As-gbr-2* gene. This would also avoid use of SL1 primer. The degenerate primer can be designed by comparison of *As-gbr-2* with sequences to which it shows high homology, particularly in the region determining the NH_2-terminal domain. Sequence comparison of derived amino acid sequences of GluCl ion channel receptor subunits, preferably from closely related parasitic nematodes such as *H. contortus* and *O. volvulus* can be used. The use of such a primer however, does run the risk of amplifying several non-specific products. A codon usage table for *A. suum* can be useful in designing the degenerate primer and making the PCR amplification more specific (Nakamura *et. al.*, 1997), (http://tisun4a.lab.nig.ac.up/codon/CUTG.html). It can keep the number of oligonucleotides in the mixture small and allows for transcription using only the preferred codons (Soppa, 1994).

5.3.3 Inverse PCR

Inverse PCR on circularised genomic DNA was used as an alternate approach for obtaining a full length 5′ RACE-PCR product. Southern hybridization analysis using *Asg2* DNA as probe was carried out on restriction enzyme digests of genomic DNA. However, no specific fragment hybridizing to the probe was noted. A possible reason for such an observation may be the complex genomic banding pattern of *A. suum*, as predicted from the nucleotide sequence of the cDNA. Geary *et. al.* (1993b) have for example reported that the restriction fragment pattern of *A. suum* genomic DNA, particularly *EcoRI*, varied extensively from what was expected when using a cDNA insert corresponding to the phosphoenolpyruvate carboxykinase gene as probe. It was

also observed that some of the restriction enzyme recognition sites absent from the cDNA resulted in several genomic DNA fragments. The *A. suum* genome in addition undergoes the mechanism of chromosome fragmentation called chromatin diminution. This could add to the complexity of the *A. suum* genome. Such observations may explain why inverse PCR amplifications were not successful.

In short, PCR amplifications of *A. suum* muscle cDNA resulted in most of the sequence of the *As-gbr-2* gene. The exact 5′ end sequence is yet to be determined. From comparison with sequences in the database, *As-gbr-2* appears to encode for a subunit that is a member of the glutamate-gated chloride ion channel family of receptor subunits. High amino acid identity of As-GBR-2 is noted with the GBR-2B subunits from *C. elegans* and *H. contortus*. Both Ce-GBR-2B and Hc-GBR-2B are encoded for by *gbr-2* mRNA that undergoes splicing. PCR amplifications of *A. suum* cDNA however suggest that the *Asgbr-2* gene may not be spliced.

IMMUNOCYTOCHEMICAL LOCALISATION OF GBR-2 SUBUNITS IN *HAEMONCHUS CONTORTUS* AND *ASCARIS SUUM*

6.1 Introduction

The anthelminthic, ivermectin has two sites of action- the nematode body-wall muscle and the pharynx. The drug is suggested to mediate its effect by opening glutamate-gated chloride (GluCl) ion channels (Wolstenholme, 1997). In *C. elegans*, coexpression of cloned receptor subunits GluClα and GluClβ in *Xenopus* oocytes results in an ivermectin sensitive channel (Cully *et. al.*, 1994). In *A. suum*, the pharyngeal muscle is shown to possess glutamate receptors susceptible to the avermectin analogue, milbemycin (Martin, 1996). Kass *et al.*, (1980, 1984) on the other hand showed that *A. suum* were immobilized by avermectin causing paralysis of the somatic musculature through action of motor neurons. In view of such observations it was considered important to further characterize the GluCl subunits cloned from *H. contortus*.

cDNAs corresponding to two GluCl ion channel receptor subunits, Hc-GBR-2A and Hc-GBR-2B were amplified by RT-PCR from *H. contortus* (Chapter 3). The subunits were encoded by a single alternatively spliced *Hc-gbr-2* gene. The derived amino acid sequence of *Hc-gbr-2* shows high identity with the alternatively spliced *gbr-2* gene from *C. elegans* (Laughton *et. al.*, 1997). A homologous *As-gbr-2* gene has also been identified in *A. suum* although it does not appear to undergo alternative splicing (Chapter 5). The derived peptide sequence of *As-gbr-2* shows high identity with sequences of the GBR-2B type of subunits. In the present study, the GBR-2 subunits were characterized further in *A. suum* and ivermectin susceptible and resistant adult *H. contortus* by immunolocalisation experiments. Polyclonal antibodies raised to a peptide matching the sequence of Ce-GBR-2B receptor subunit in the NH2-terminal domain were used.

6.1.1 Peptide used to raise polyclonal antibodies

A 13 amino acids long peptide, Gbrpep, was synthesized on a Milligen 9050 Pep-synthesizer. The peptide sequence $_1$ARVVLRLRREYSC$_{13}$ matched a region just upstream

from transmembrane I of Ce-GBR-2B (Appendix II). The peptide was coupled to a carrier protein thyroglobulin by the m-maleimidobenzoic acid N-hydroxysuccinimide ester (MBS) method by Horoszok, L. (Marrion, 1949). New Zealand white rabbits were subsequently immunized with the thyroglobulin-peptide conjugate and ELISA tests carried out on pre immune serum and serum from test bleeds to monitor the antibody titre. Serum was finally harvested and stored for further use.

Sequence of the Gbrpep peptide was aligned with other GluCl ion channel receptor subunits to determine the extent of sequence conservation in this region. Highest similarity in sequence was observed with the derived amino acid sequences of *Hc-gbr-2* and *As-gbr-2* (Figure 6.1). Only two substitutions of $_4$Val with Lys and $_6$Arg with Leu were noted in both Hc-GBR-2B and As-GBR-2. Since a 85% match occurred between the sequences, and since the antibodies raised to the peptide antigen were polyclonal, the serum was used for immunolocalisation of the GBR-2 subunits in both *H. contortus* and *A. suum*.

Affinity purification of GBR-2 specific antibodies from the polyclonal serum was carried out on a peptide antigen column. An ELISA test was done to determine antibody titre before immunolocalisation of adult *H. contortus* and *A. suum*. The whole mounts were finally examined by confocal microscopy.

Ce-GBR-2B	ARVVLRLRREYS
Hc-GBR-2B	ARVKLLLRREYS
As-GBR-2	ARVKLLLRREYS
Ce-GBR-2A	LRTRMVLRREFS
Hc-GBR-2A	LRTQMVLRREFS

Figure 6.1 Sequence alignment of GBR-2B receptor subunits in the region matching the Gbrpep peptide (designed to match the Ce-GBR-2B sequence). Amino acid residues differing between the sequences are underlined.

6.2 Results

6.2.1 Affinity purification of antibody on an antigen column

Polyclonal anti-Gbr2bpep antibodies were purified on an antigen column prepared by covalently coupling the peptide to a solid support Sepharose matrix (Section 2.14.1). Specific antibodies bound to the antigen upon addition of the antiserum to the column. Non-specific antibodies were washed away with phosphate buffer (pH 7.3). Antibodies were finally eluted under high alkaline pH (10.7) using diethylamine. The elution process was monitored by passing the sample though an optical ultraviolet unit, calibrated at first using 10mg/ml Bovine Serum Albumin (BSA) in phosphate buffer (pH 7.3). Antibody eluted from the column showed up as a peak at absorbance 280nm. The eluant was collected as the peak started to appear. A final eluant collection volume of 35ml was dialysed and concentrated to a smaller volume of 1.6ml using PEG 20,000. Antibody titre was then determined using the Enzyme-Linked Immunosorbent Assay (ELISA) (Section 2.14.2).

6.2.2 ELISA tests

96 well microtitre plates were coated with 100µl of the Gbrpep antigen (10µg/ml). The assay was carried out using 1) buffer as control, 2) pre-immune serum, and 3) affinity column purified antibody. Anti-goat rabbit peroxidase conjugate was used as secondary antibody. Catalysis of the peroxidase enzyme finally resulted in a coloured product (See Web page- http://www2.perkin-elmer.com/pa/340913/html). OD^{450} of the coloured reaction products were read for the samples on a ELISA plate reader. Absorbance and antiserum dilution were plotted on a graph and the inflection point of the post-immune serum determined (Figure 6.2). Serum titre against the peptide assayed resulted in a half maximal response at 1:500 dilution.

6.2.3 Immunostaining of ivermectin susceptible and resistant *H. contortus*

Adult female and male ivermectin susceptible *H. contortus* were fixed in 4% (v/v) PFA in PBS and permeabilised with 120 collagen digestion units (CDU)/ml of collagenase in collagenase buffer for 10h (See Section 2 (D). This appeared to result in increased cuticular digestion, causing the heads to break off from the rest of the body. Samples were

therefore collagenase treated with 115 CDU/ml for 8h. Worms were processed further for immunostaining, using Gbrpep specific primary antibody. As a low antibody titre was observed in ELISA, this was used neat, or in 1:5 and 1:10 dilutions. TRIT-C conjugated anti-rabbit IgG was used as secondary antibody. Worms were mounted on slides and examined by epifluorescence confocal microscopy (Section 2.16.1). A Zeiss LSM 510 confocal fitted to an inverted Axiovert 100M confocal microscope, using a helium neon laser and rhodamine filter set was used.

No specific staining was noted when neat antibody was used. Staining with 1:10 dilution of antibody was on the other hand faint suggesting that serum titre against native Gbr2 was low. Best staining was observed in the presence of a 1:5 antibody dilution.. The majority of the staining was concentrated in the central portion of the worm (Figure 6.3A). Specific staining of the nerve cord and motor neuron commissures is noted Figure 6.3C). The negative control using a vast excess of the peptide showed no staining (Figure 6.3B). A second negative control in the absence of the primary antibody gave similar results. Figure 6.3D shows staining of what may be interneurons. An animation series of the confocal images of commissural branches arising from the ventral cord to traverse the circumference of the animal and approach the dorsal nerve cord is shown in Figure 6.4. The nerve cord was identified by comparison of structures staining with Gbrpep specific antibody with descriptions of the motor nervous system by Johnson and Stretton (1987) for *A. suum*. The confocal images were collected at different projections. Staining was also noted in the nerve ring (Figure 6.5, 6.6) and a region which may be the dorsal lateral cord. Some staining was noted in the tail regions of worms of both sexes (Figure 6.7). No pharyngeal staining was observed.

Adult ivermectin resistant *H. contortus* were also examined in order to localise the GBR-2 receptor subunits. Collagenase treatments of 8h, 10h and 12h did not result in any staining. As the worms physically appeared robust when compared to the ivermectin susceptible *H. contortus*, an extended collagenase treatment was expected to result in some staining. The worms were therefore treated with 115 CDU/ml for 15h. A 1:5 dilution of the primary antibody was used. Staining similar to that observed in ivermectin susceptible *H. contortus* worms was seen.

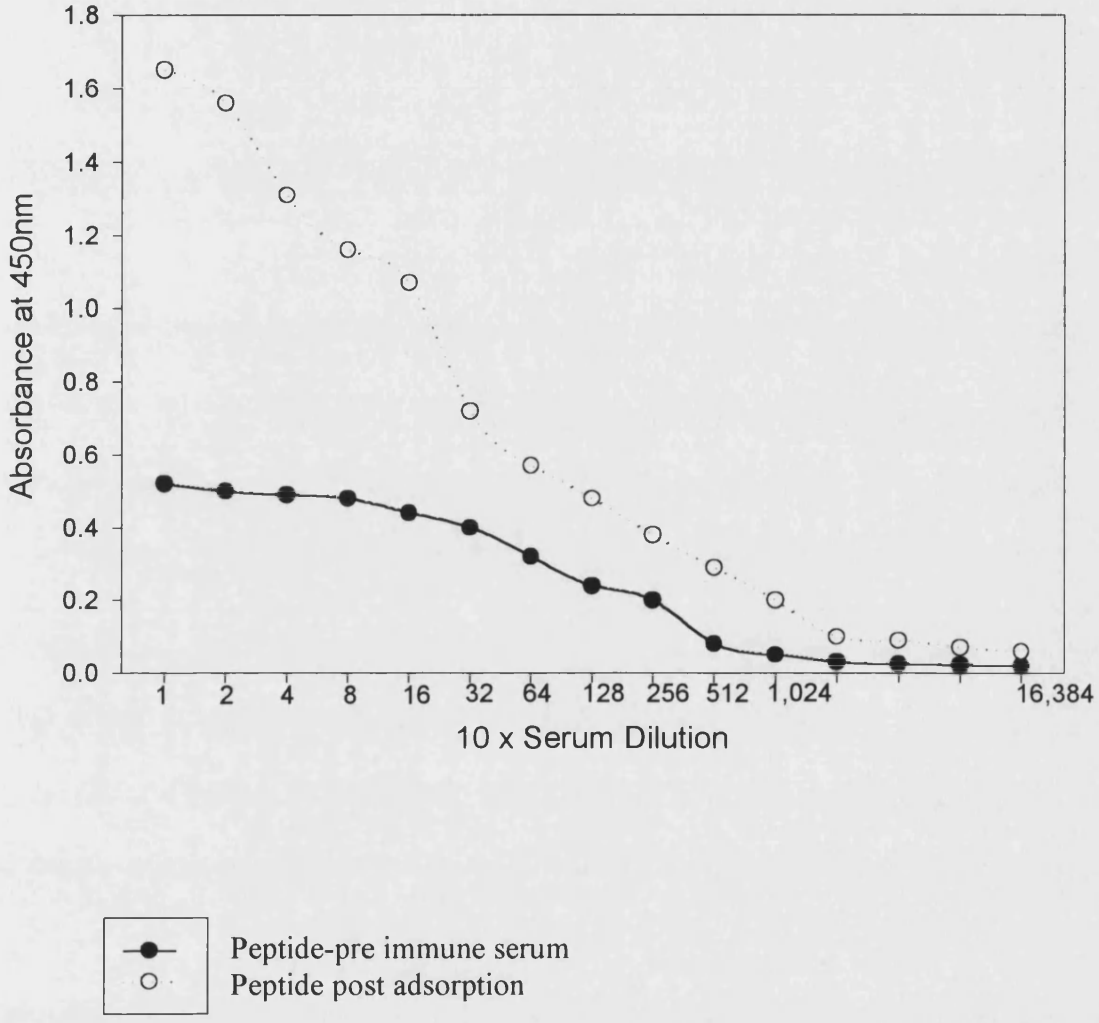
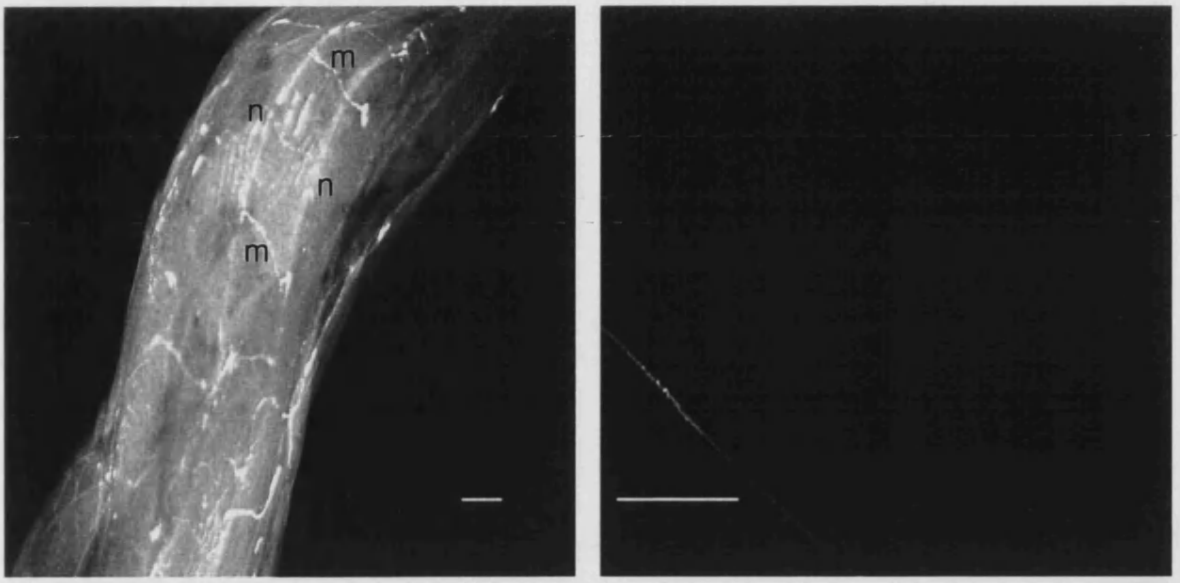


Figure 6.2 Antibody titre graph to analyse ELISA results of antiserum against GBR-2 specific antibody.

Figure 6.3 Immunolocalisation of GBR-2 in adult *H. contortus*. **(A)** Confocal image of *H. contortus* staining with anti-GBR-2 antiserum at a 1:5 dilution of the nerve cord (n) and motor neuron commissures (m). The worm is dorso-ventrally compressed. **(B)** High resolution image of negative control using antiserum pre-adsorbed with 4μM Gbrpep peptide one hour before incubation. **(C)** High resolution confocal image of motor neuron commissures. **(D)** High resolution image showing possible staining of interneurons (i). Scale Bar=50μm.



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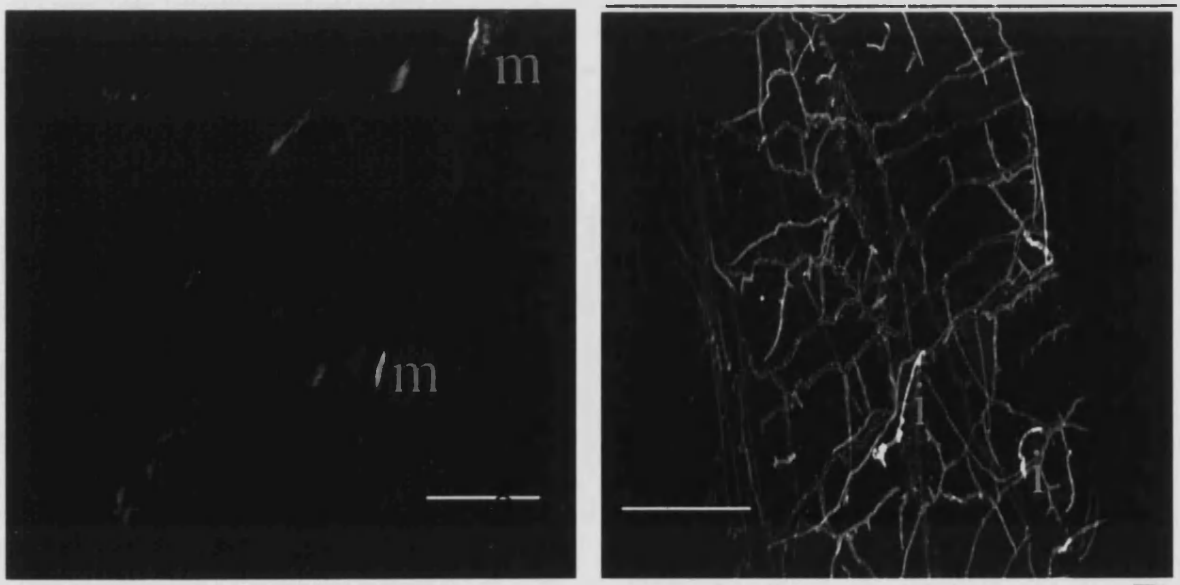
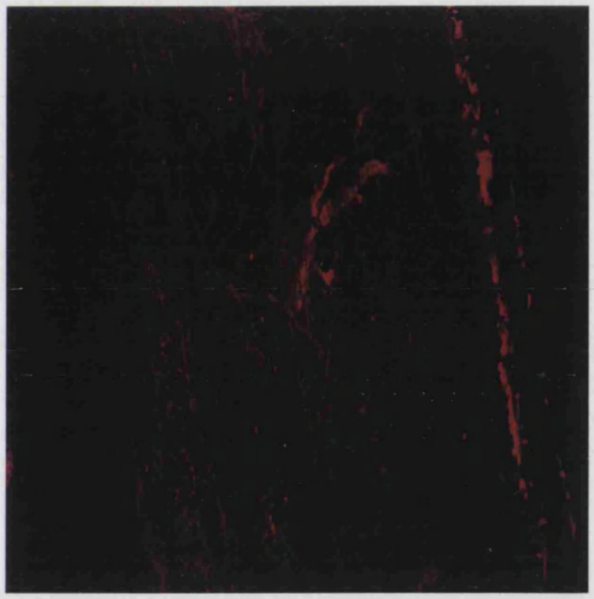


C D

Figure 6.4 Confocal animation series of the motor neuron commissures in *H. contortus* staining with GBR-2 antiserum. **(A)-(D)** Commissure (m) arising from the ventral cord to form a neuronal branch. During animation, the specimen is apparently rotated by a certain angle from image to image by a full turn about an axis. If such a sequence is displayed on the monitor screen in rapid succession, the visual effect is that of rotating a three-dimensional object. **(E)** The entire set of data A-D is imaged as a single projection. The image produced by this method has a virtually infinite depth of focus as the result constitutes information only from in-focus planes. Staining of possible interneurons (i) can be seen. Scale Bar=50μm.

A



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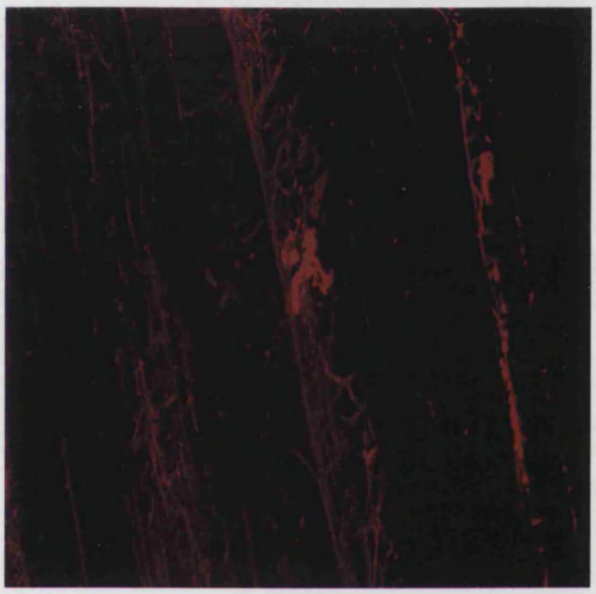

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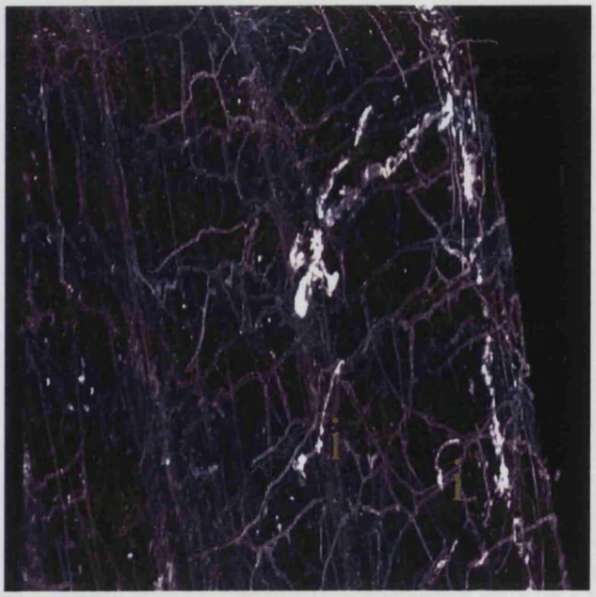
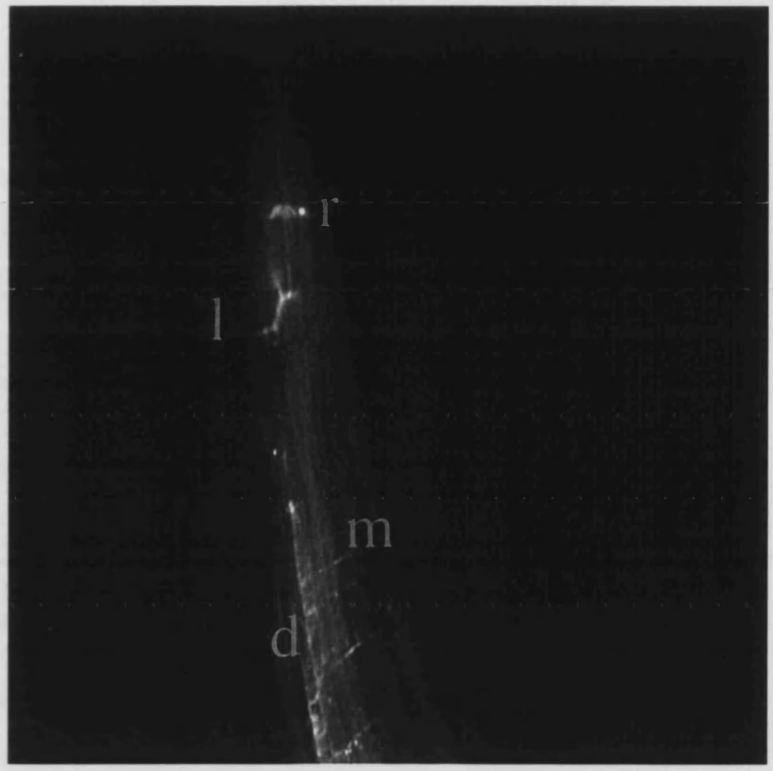


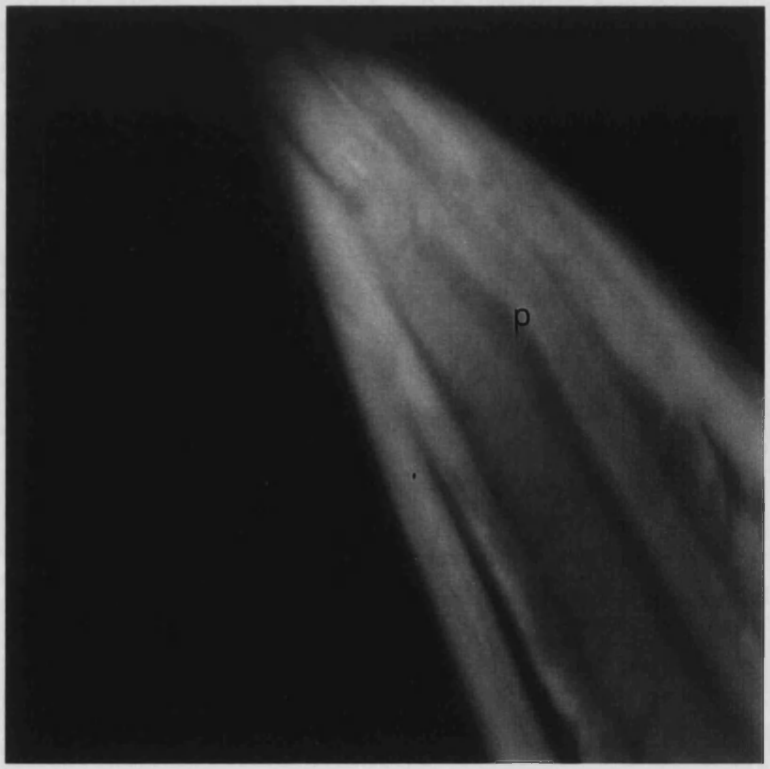
E

Figure 6.5 **(A)** Confocal image of nerve ring (r) staining in adult *H. contortus* incubated in a 1:5 dilution of anti-GBR-2 antiserum. Staining of motor neuron commissures (m) traversing the body to join the dorsal cord (d) can be seen. Projecting towards the nerve ring is possibly the dorsal lateral cord (l). **(B)** Confocal image under high resolution of the negative control using antiserum pre-adsorbed with 4μM Gbrpep peptide prior to incubation. The pharynx (p) is seen as a long tube. Scale Bar=50μm

Figure 6.6 Confocal image of staining of the nerve ring (r) under higher resolution. Scale Bar=50μm. (See Page 160)



A



B

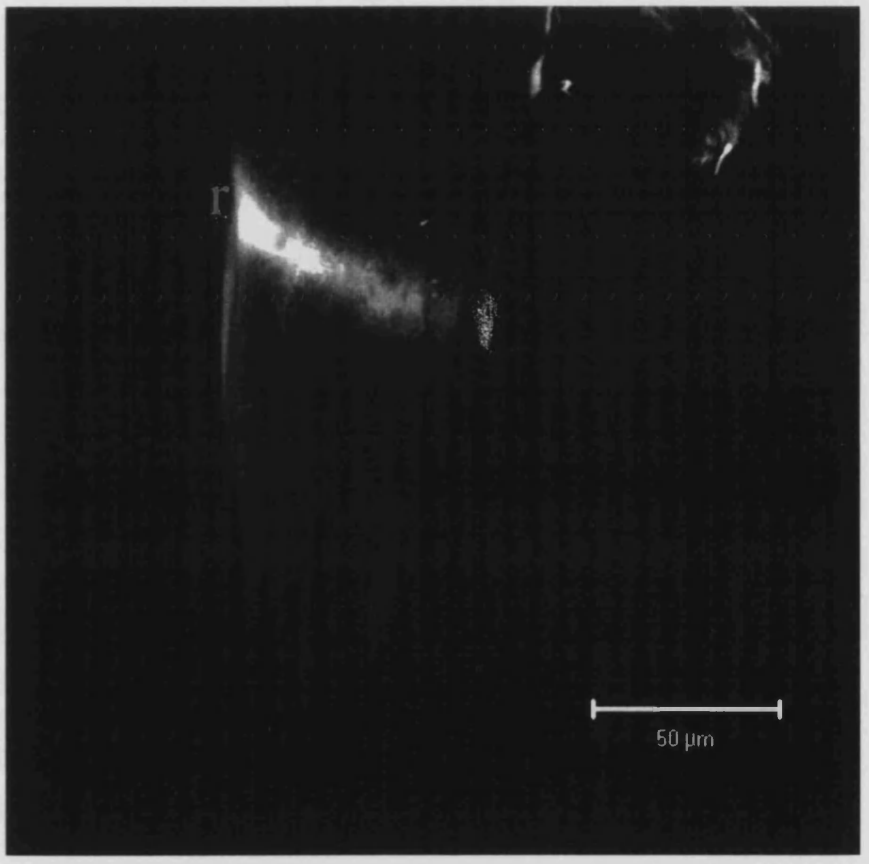
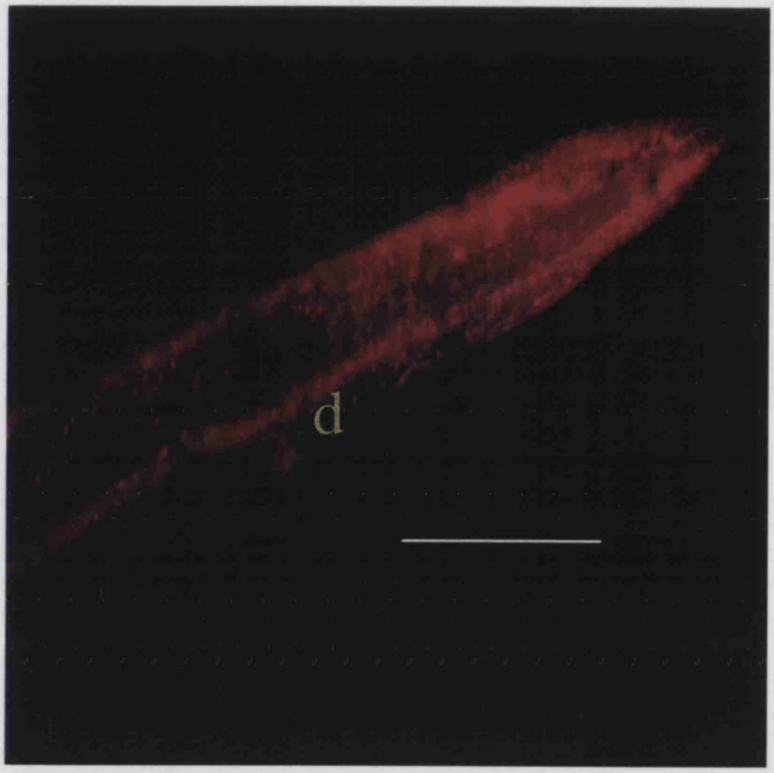
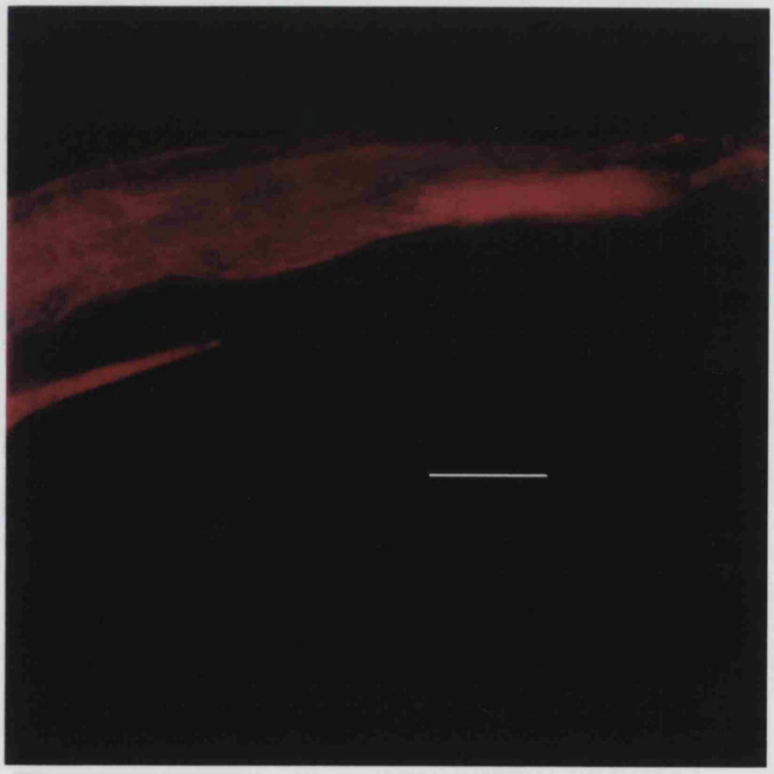

r
50 µm

Figure 6.7 **(A)** Confocal image of GBR-2 immunoreactivity in adult female *H. contortus* tail. Staining of the dorsal nerve cord (d) can be seen at a 1:5 dilution of anti-GBR-2 antiserum. **(B)** Negative control using antiserum preadsorbed with excess Gbrpep peptide before incubation. Scale Bar=50μm.



A



B

6.2.4 Immunostaining of *A. suum*

6.2.4.1 Cryosections

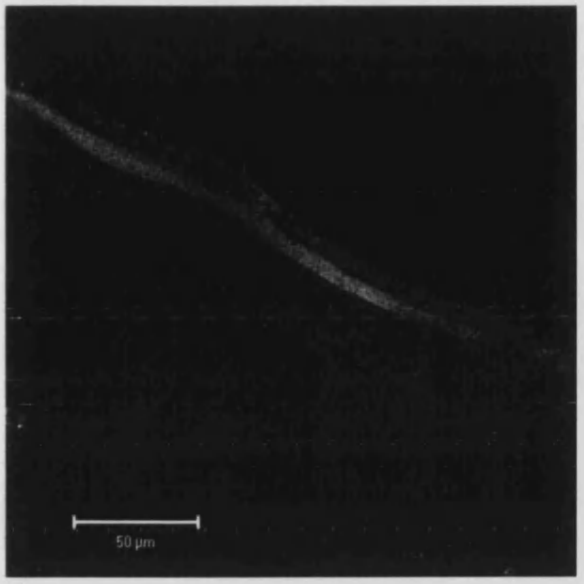
Cryosections of adult *A. suum* fixed in 4% (v/v) PFA in PBS were processed for immunostaining (Section 2.16.2). 20μm thick sections of 1cm of *A. suum* head were examined (Angstadt *et. al.*, 1989). A 1:5 dilution of anti Gbrpep antibody was used. Sections were examined under the confocal microscope for staining. The cuticle was found damaged in some sections and the background staining high. Slides were treated with 5% goat serum as a blocking agent in an attempt to reduce background staining and then processed. However this did not improve results. An alternate method of staining of whole mounts was therefore chosen.

6.2.4.2 Whole mounts

Body wall sections and muscle cells from collagenase treated *A. suum* were processed for immunostaining using Gbrpep specific antibody at 1:50 and 1:10 dilutions. TRIT-C conjugated anti-rabbit IgG was used as secondary antibody. Samples were processed further as described in Section 2.16.3 and examined by epifluorescence confocal microscopy. Specific staining was found on both the dorsal and ventral nerve cords at 1:10 primary antiserum dilution (Figure 6.8). The staining was limited to a nerve bundle on one end of the cord. Nerve cords were identified during dissection, the ventral cord being slightly thicker than the dorsal cord. Specific staining was also observed in what appears to be a dorsal sublateral nerve cord (Figure 6.9). No staining was seen with pre-adsorbed antiserum or in the presence of the secondary antibody alone. No staining of the muscle cells was seen.

Figure 6.8 Confocal images of the staining of *A. suum* nerve cord. Staining of the **(A)** dorsal cord. **(B)** ventral cord. **(C)** DIC (Differential interference contrast) image of the dorsal cord. **(D)** DIC image of the ventral cord. **(E)** Negative control using antiserum pre-adsorbed with an excess of Gbrpep peptide prior to incubation. Scale Bar=50μm.

Figure 6.9 **(A)-(B)** Confocal image of staining observed in the dorsal sub-lateral cord (s). **(C)** DIC image under higher resolution. **(D)** DIC image showing the dorsal cord (d) running parallel to the dorsal sub-lateral cord. Scale Bar=50μm.



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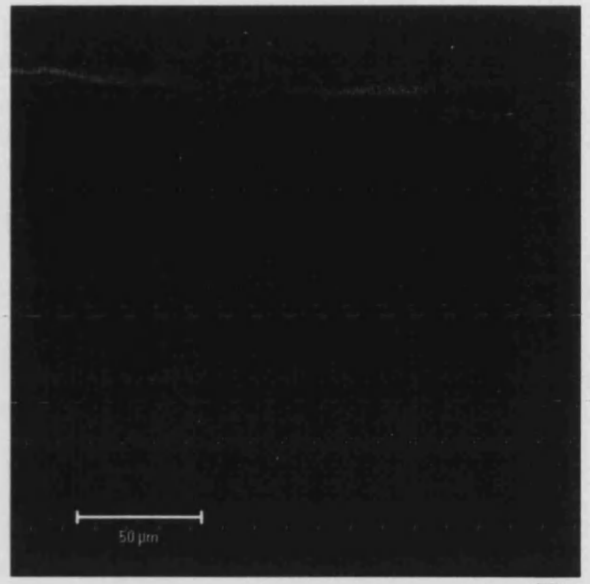
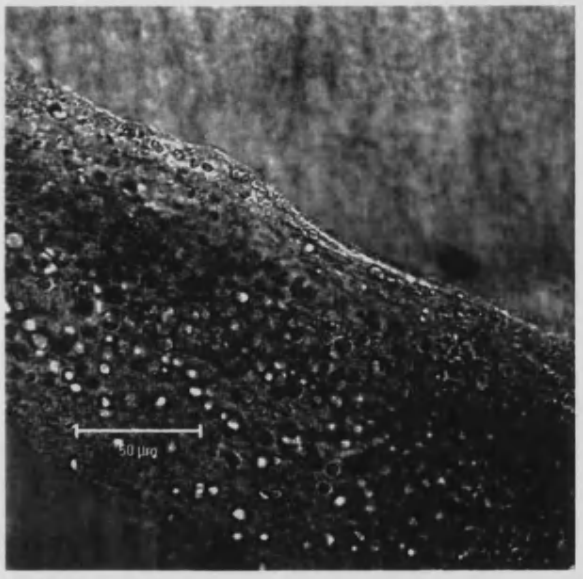


B



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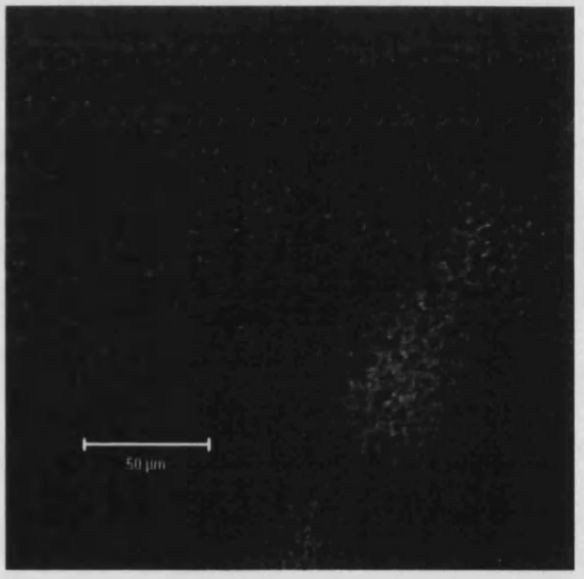
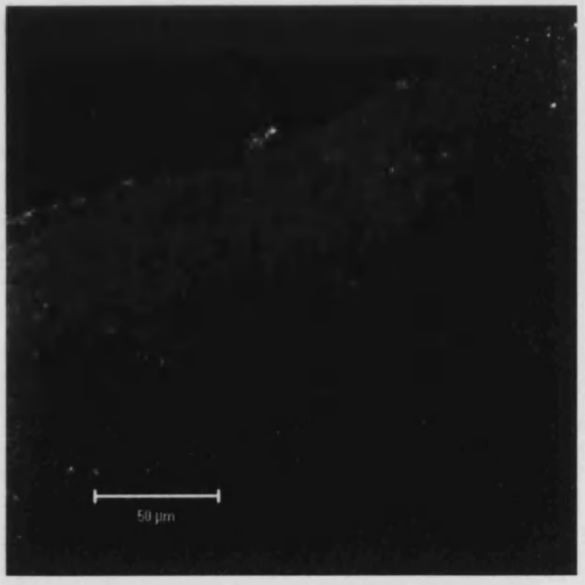


D



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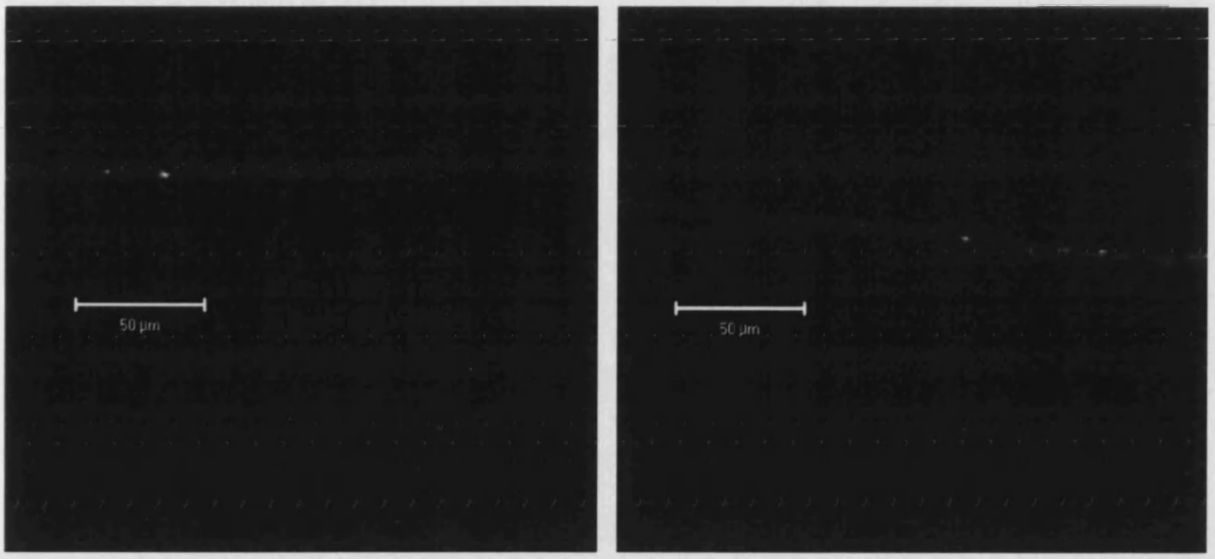
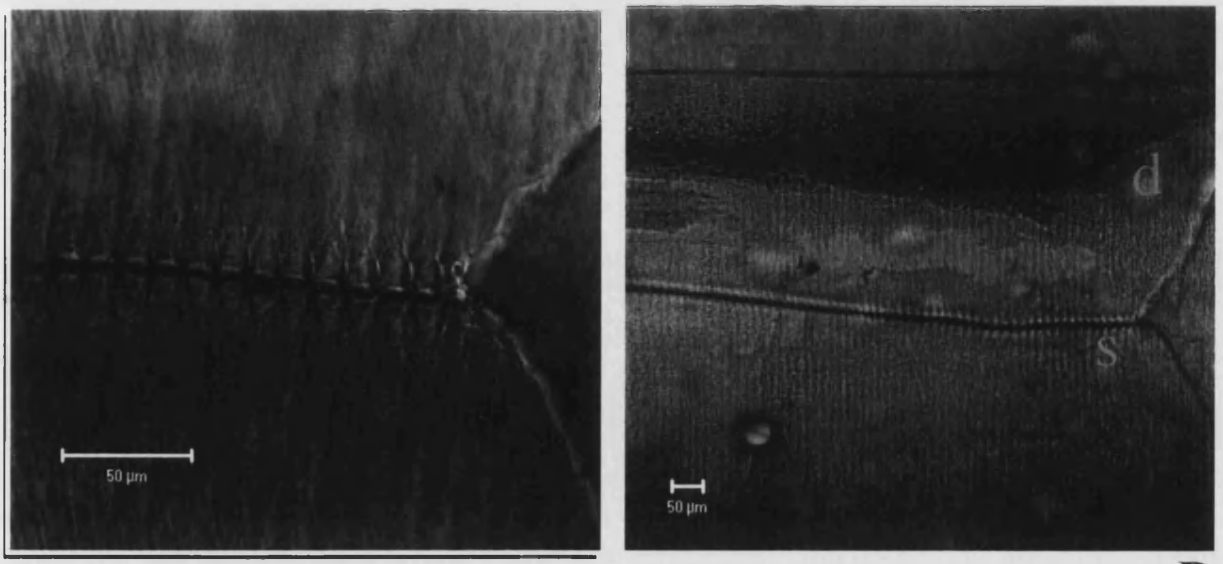


A B



C D

6.3 Discussion

The Gbrpep peptide designed originally to the NH_2-terminal region of the predicted *C. elegans* GBR-2B subunit showed similarity in its sequence with the GBR-2 subunits from *H. contortus* and *A. suum*. No sequence homology in this region was observed with other members of the GluCl family. Given the strong conservation in sequence between the GBR-2 type of subunits, antibodies to Gbrpep were expected to show specific staining in *H. contortus* and *A. suum*. However, the antibodies would not distinguish between GBR-2A and GBR-2B which exhibit high amino acid identity in their sequence. Specific staining was observed in both sexes of *A. suum* and ivermectin susceptible and resistant adult *H. contortus* during localisation experiments of the GBR-2 receptor subunit. In *H. contortus* this was noted in motor neuron commissures, nerve cords and the nerve ring. No pharyngeal staining was seen. In *A. suum*, nerve cord staining was observed. There was no staining of the muscle cells. Pre-adsorption of the antiserum with the synthetic peptide blocked all specific staining, confirming that the immunoreactivity was due to the presence of the receptor subunit and not to cross-reacting antigens.

The localisation results suggest that although the antisera used was not ideal, it can be used to determine the expression patterns of subunits with closely related sequences. The technique may be applied for other antibodies as well. Use of antibodies specific to Hc-GBR-2A or 2B or As-GBR-2 would on the other hand, avoid background staining and also clearly distinguish between the 2A and 2B type of subunits.

6.3.1 Structural comparison of *H. contortus* with *A. suum*

Very little data is available on the neuromuscular system of *H. contortus*. Structural comparisons were therefore made with *A. suum*. The nervous system of *A. suum* mainly consists of a series of ganglia in the head associated with the nerve ring. Two major nerve cords, the dorsal and ventral cords originate in the nerve ring and run along the animal to the tail, where a second smaller set of ganglia are present. The nerve cords send out branches of neurons called 'commissures' in this region. Each commissure constitutes a single motor neuron and is buried within the thin layer of hypodermal tissue, just beneath the cuticle. The commissures either traverse to the left side or the right. These have been functionally classified by Stretton *et. al.*, (1978). From the immunolocalisation

results in *H. contortus*, the staining appears to correspond to the neuronal structures described for *A. suum*. In Figure 6.3A in which the worm appears to be dorso-ventrally compressed, staining of nerve cords is visible. Neuronal branches traversing specifically in either direction, most likely of motor neuron commissures also show staining. An animated confocal image series in which the structure is rotated in both horizontal and vertical axes shows more detail of a neuronal branch (Figure 6.4). Staining of the nerve ring is also prominent as seen in Figure 6.6. Projecting forward towards the nerve ring is possibly the dorsal lateral cord which also shows some staining. A similar structure is visible in *A. suum* (Johnson and Stretton, 1987).

Nerve cord staining was also noticed in the caudal region of adult male and female *H. contortus*. However no staining of any specific ganglia were apparent. Staining of the caudal region may be relevant. Neurons in this region of the *A. suum* male tail for example, have been shown to result in serotonin specific immunoreactivity. Serotonin is suggested to influence locomotion and mating posture (Johnson *et. al.*, 1996; Reinitz and Stretton, 1996).

6.3.2 Possible mechanism of action of ivermectin in *H. contortus*

Pharmacological and physiological studies on the action of ivermectin and its analogues show that these drugs act on the nematode body-wall muscle or pharynx. In *A. suum*, Kass *et al.*, (1980, 1984) have shown that nematodes were immobilized by paralysis of the somatic musculature. The action of avermectin was mediated by interneurons and inhibitory motor neurons. More recently, attention has shifted to the pharynx as a site of paralysis caused by ivermectin and its analogues as shown in *A. suum* (Brownlee *et. al.*, 1997). Inhibition of pharyngeal pumping in *H. contortus* is also reported to occur more potently than the inhibition of motility (Geary *et. al.*, 1993).

There is also growing evidence that the avermectin anthelminthics act via glutamate-gated chloride (GluCl) ion channels (Martin *et. al.*, 1997). Two functional cDNAs GluClα and GluClβ isolated from *C. elegans*, when expressed in *Xenopus* oocytes, encoded for glutamate-gated and ivermectin-sensitive chloride channel subunits (Vassilatis *et. al.*, 1996). cDNA clones of other members of the GluCl family such as the alternatively spliced *gbr-2* gene, encoding for two subunits GBR-2A and GBR-2B, have also been isolated from *C. elegans* (Laughton *et. al.*, 1997). This is supported by recent

pharmacological data using the ivermectin resistant strain, *avr-14* of *C. elegans* (Grant and Johnson, *pers. comm.*). The presence of glutamate receptors gating chloride channels that potentiate ivermectin has also been shown in *A. suum* (Martin, 1996).

The immunolocalisation results in *H. contortus* appear to confirm the findings in *C. elegans* and *A. suum* to an extent. Localised mainly in the nerve cords, the motor neurons, and the nerve ring, this result compares well with the observations of Kass *et. al* (1980) in *A. suum* . They show that avermectin B1a paralyses the worm by acting on interneurons and motor neurons. This also connects well with the observation that a majority of the neurons in *H. contortus* are stained in the central region of the body (Delany, N., *pers. comm.*). Geary *et. al.*, (1993) and Gill *et. al.*, (1995) observe in *H. contortus* adults and larvae respectively that ivermectin induced paralysis was restricted to the mid-body region of the parasite, while the head and tail sections had normal motility.

Interestingly, no pharyngeal expression was noted. This is in contrast to several observations of the expression of GluCl receptor subunits in the pharynx. GluClβ and the alternatively spliced GluCl-α2A and 2B receptor subunits corresponding to the *avr-15* ivermectin resistance strain in *C. elegans* are expressed in the pharyngeal muscle (Dent *et. al.*, 1998; Laughton *et. al.*, 1997). There is evidence that the Ce-GBR-2B receptor subunit encoded by the *Ce-gbr-2* gene is localized in the pharynx (Horoszok. L., *pers. comm.*).

Hc-GBR-2B expression however, compares well with the expression observed for another GluCl receptor subunit, HG4, in *H. contortus*. The cDNA clone of HG4 shows high sequence identity with GluClβ in *C. elegans*. But, in contrast to the pharyngeal expression of GluClβ, HG4 is expressed in motor neuron commissures, with some expression on the nerve cords (Delany. N., *pers. comm.*).

6.3.3 Immunostaining of *Ascaris suum*

Staining in cryosections of *A. suum* head did not appear very specific. It is possible that the lack of discrete staining was due to the choice of the method used for study. The use of whole worm preparations could give better results. However, a negative result in staining would be observed if the As-GBR-2 receptor subunit was not expressed in the head or pharyngeal region. This could at least in part explain the difficulty in identifying an *As-gbr-2* clone from the cDNA library constructed from *A. suum* heads (Chapter 4).

In whole worm preparations, As-GBR-2 was localised to the nerve cords. Staining was also observed on what may be the dorsal sublateral nerve cord. Nerve cord staining in *A. suum* compares well with the observations made in *H. contortus*. The results suggest that in addition to conserved GBR-2 sequences in *H. contortus* and *A. suum*, their expression patterns are also comparable to an extent.

In summary, the GBR-2 type of glutamate-gated chloride (GluCl) ion channel receptor subunit has been localised in *H. contortus* and *A. suum*. It is expressed in the nerve cord, motor neuron commissures and nerve ring of *H. contortus*. No pharyngeal expression is observed. The nerve cords stain in *A. suum*. The GluCl ion channel may serve as a putative site for the action of the anthelminthic, ivermectin.

7. FINAL DISCUSSION

The family of inhibitory glutamate receptors is closely related to ionotropic glycine and GABA receptors, but is confined to invertebrates. Glutamate gates the receptors directly and opens channels permeable to chloride ions (GluCl). Two GluCl receptor subunits, GluClα (now called GluClα1) and GluClβ were initially cloned from the free-living nematode *C. elegans* (Cully *et. al.*, 1994). The α1 subunit forms ivermectin-gated chloride ion channels and the β subunit forms GluCl channels when expressed in *Xenopus* oocytes. The α1 subunit also possesses a cryptic glutamate-binding site which is not coupled to the opening of the channel (Etter *et. al.*, 1996). Coexpression of the subunits result in an ivermectin potentiated GluCl. Two other inhibitory glutamate receptor subunit cDNAs, *Ce-gbr-2a* and *Ce-gbr-2b*, encoded by an alternatively spliced gene have been cloned from *C. elegans* (Laughton *et. al.*, 1997). The derived peptide sequences showed about 55% and 50% amino acid identity with GluClα1 and GluClβ respectively. Another alternatively spliced gene *GluClα2* encodes two subunits 2A and 2B. Its putative ORF bears 83% amino acid identity with GluClα1. Several other related GluCl receptor subunits have also been identified from the *C. elegans* genome sequencing project.

The present study has involved PCR amplification and sequence analysis of cDNAs from parasitic nematodes *H. contortus* and *A. suum* encoding for orthologues of the *Ce-gbr-2* gene. The *gbr-2* gene from *H. contortus* is alternatively spliced. It encodes two subunits, Hc-GBR-2A and 2B as a result of a partial gene duplication event (accession numbers HCY14233 and HCY14234). A full-length mRNA, 1997 nucleotides long encodes the Hc-GBR-2A subunit. This mRNA species has a 731 nucleotide long 3′ untranslated region which contains the channel encoding sequence of the Hc-GBR-2B subunit. A spliced mRNA 1317 nucleotides long encodes for the Hc-GBR-2B subunit. The GBR-2A channel encoding domain is absent in this mRNA. Both *Hc-gbr-2a* and *2b* mRNAs possess a common 735 nucleotides long region which encodes the NH_2-terminal domain. This may result in identical ligand-binding sites for the two subunits. However, the channel determining regions exhibit only 57% amino acid identity with each other, suggesting that slightly different chloride channels may be encoded for.

The derived peptide sequences of Hc-GBR-2A and 2B subunits exhibit 88% and 83% amino acid identity with Ce-GBR-2A and 2B respectively. Amino acid residues in the TM II membrane-spanning domain lining the ion channel are identical between Ce and Hc GBR-2A subunits. But two residues differ in this region when compared with the 2B receptor subunits- Ce-Thr $_{287}$, Ser $_{300}$ and Hc-Thr $_{295}$, Ser $_{308}$ in 2A are substituted with Ce-Ser $_{287}$, Ala $_{300}$ and Hc-Ser $_{295}$, Ala $_{308}$ in 2B. These substitutions are particularly striking as Thr_{287}/Ser_{308} is equivalent to the amino acid residue (Ala→Ser) corresponding to a resistance associated point mutation in the GABA receptor of field isolated *Drosophila melanogaster* mutant, *Rdl* (resistant of dieldrin) (ffrench-Constant *et. al.*, 1993a, b). Mutations at this position (Thr→Pro/Gly/Ala) also enabled glutamate gating of GluClα1 homomeric channels (Etter *et. al.*, 1996). The TM IV membrane-spanning domains of Ce and Hc-GBR-2A are identical; Ce and Hc-GBR-2B are 70% similar in this region.

Hc-gbr-2a and *2b* cDNAs were PCR amplified from ivermectin resistant isolates of *H. contortus*. No differences in the sequence at the amino acid level were observed when compared to the *Hc-gbr-2* sequence from the ivermectin susceptible isolate. Changes in the nucleotide level were however noted, possibly occurring as a result of polymorphic variations. The *gbr-2a* and *2b* mRNAs in eggs from ivermectin susceptible *H. contortus* were also compared for their relative expression levels by a semi-quantitative PCR analysis. The *gbr-2a* mRNA was expressed at much lower levels than *gbr-2b*. This differed from *Ce-gbr-2a* and *2b* mRNAs isolated from a developmentally mixed population of *C. elegans* which exhibited similar expression levels as determined from Northern blot analysis (Laughton *et. al.*, 1997). Differences in ratio of the expression levels of the two *gbr-2* mRNAs in both species could suggest differences in the regulation of the alternative splicing pathways or a means of regulating the availability of the number of mRNA copies encoding for functional GBR-2A and 2B receptor subunits. Similar differences in expression levels were noted between the two mRNAs in the ivermectin resistant isolate of *H. contortus*. Similar quantitative PCR analysis of the two mRNAs in larvae and adults could demonstrate if altered expression levels is developmentally regulated.

The *Ce-gbr-2* gene is found to map very close to the *avr-14* gene for resistance to ivermectin in *C. elegans* (Dent and Avery, 1998). It encodes for the alternatively spliced GluCl receptor subunits, GluClα2A and GluClα2B. *avr-14* maps close to another gene

avr-15, which also confers high-level synthetic resistance to ivermectin. GluClα2A exhibits 85% amino acid identities with GluClα1 and responds to both glutamate and ivermectin. Thus several members of the family of GluCl receptor subunits identified in *C. elegans* appear to serve as important targets of ivermectin. Common features between the sequences and splice patterns of the *Ce-gbr-2*, *Ce-Glucl*α*2* and *Hc-gbr-2* mRNAs suggests that similar *avr* genes and GluCls are likely to occur in *H. contortus*. It is also likely that the presence of more than one alternatively spliced *GluCl* gene increases the number of subunits and possible subunit combinations available to the nematode.

Expression of subunits Hc-GBR-2A and 2B in *Xenopus* oocytes independently, and their coexpression would therefore prove useful in determining the type of homomeric /heteromeric channels possible and the ligands responsible for opening these channels. The GBR2A and 2B subunits can also be expressed along with other GluCls, the GluClα-like (HG5) and GluClβ-like (HG4) subunits identified in *H. contortus* (Delany, N., *pers. comm.*).

A second part of this study has involved PCR amplification of the *As-gbr-2* gene from dissected *A. suum* muscle. This is the first report of a receptor sequence in *A. suum* of the ligand-gated chloride channel family. Comparison of the derived peptide sequence of the As-GBR-2 subunit in the database with other sequences shows that it is a member of the GluCl family. The sequence exhibits highest amino acid identity with the GBR-2B type of subunit- 82% and 83% with Ce and Hc-GBR-2B subunits; and 74% with Ce and Hc-GBR-2A subunits respectively. The sequence of the TM II membrane spanning domain of As-GBR-2 is again identical to subunits Ce and Hc-GBR-2B; sequence in the TM IV region shows 74% similarity to Hc-GBR-2B and 65% to Ce-GBR-2B. As observed from sequence comparison, it is possible that the *As-gbr-2* gene is also an orthologue of the *gbr-2* genes from *C. elegans* and *H. contortus*. However, PCR amplification from cDNA in the region with the potential splice site resulted in only a single product, suggesting that the *As-gbr-2* gene is not alternatively spliced. Attempts to confirm this observation from genomic PCR amplifications met with little success, probably due to the complexity of the *A. suum* genome and its restriction digest pattern. cDNA sequences obtained were repeatedly truncated in several PCR amplifications carried out by altering various conditions. One explanation for

obtaining truncated products may be the complex splicing machinery involved in nematodes, particularly in *A. suum*.

In order to obtain the full-length sequence of *As-gbr-2*, a cDNA library was constructed from *A. suum* heads. However, extensive screening for the *As-gbr-2* gene did not yield a positive result. This could suggest either the representation of the *As-gbr-2* mRNA species in a very low copy number, or, perhaps even its absence from the head region. Localisation experiments demonstrate expression of the As-GBR-2B subunit in other regions of the body. However, this does not necessarily rule out its expression in the pharynx or the head region as localisation studies were carried out using whole mounts of body wall sections dissected immediately after the head region. Obtaining the full-length sequence of the As-GBR-2 subunit can be vital as this can be compared with the growing pharmacological data available on the presence of GluCl receptors in *A. suum*.

Immunolocalisation of the GBR-2 subunits on whole mounts of both *H. contortus* and *A. suum* were carried out using an antiserum raised against a twelve residue long synthetic peptide. The peptide was designed to match the sequence of the Ce-GBR-2B extracellular domain and conjugated to thyroglobulin to increase its immunogenicity by L. Horoszok. The derived amino acid sequences of the GBR-2 subunits exhibited a high 80% sequence similarity in this region

GBR-2 specific antibodies were purified on an affinity column. Serum titre against the peptide assayed using the ELISA resulted in a half maximal response at 1:500 dilution. The majority of the staining in adult *H. contortus* was observed in the motor neuron commissures and the dorsal and ventral cords. This compares well with the structure of the motor neurons in *A. suum* in which neuromuscular synapses for five of the seven segmental neurons have commissures in the dorsal cord. Staining was also observed in the nerve ring, with some in the caudal region. Similar expression was observed in ivermectin resistant isolates of *H. contortus*. In *A. suum* again, staining of both the dorsal and ventral nerve cords, and possibly the dorsal sub-lateral cord was observed. No staining was noted on muscle cells. The localisation results appear to compare in part with the observations made for the avr-14 gene in *C. elegans* . *avr-14* gene expression was noted in a subset of ring neurons and two neurons in the tail, but not in the pharynx.

The identification and characterisation of orthologues of the *C. elegans* GluCls in adult parasitic nematodes, *H. contortus* and *A. suum* suggests that these receptor subunits

are widely conserved among nematodes. This is further supported by successful immunolocalisation of the GBR-2 receptor subunits on two different parasitic species using antisera raised against a synthetic peptide designed to match the sequence in *C. elegans*. However, in order to prevent any cross reactivity, localisation is best done using subunit specific antisera, preferably raised to the intracellular loop region unique to each subunit. This would distinguish clearly between GBR-2A and GBR-2B subunits, and also other GluCl subunits occurring as a result of alternative splicing. On the other hand, the subunits can be localised by reporter gene constructs of GFP (green fluorescent protein) fusions in the intracellular loop region of GBR-2A and 2B subunits.

Expression of the GBR-2 GluCl subunits in a major part of the nervous system of both parasitic nematodes *H. contortus* and *A. suum*, coupled with the data available on the response to ivermectin observed for other channels of the GluCl family could suggest the potential of these subunits as drug targets. The localisation data particularly supports the observations of Kass *et. al.*, (1980) in *A. suum* who show that ivermectin paralyses the worm by acting on motor neurons. A model involving both the pharynx and the nervous system of the worm in response to ivermectin is shown in Figure 7.1. It is possible that the action of ivermectin via paralysis of the pharynx or the motor nervous system depends upon the nematode species. The exact mechanisms however, need to be elucidated. Although the GluCls of parasitic nematodes exhibit sequence similarities to those in *C. elegans*, they differ in their localisation and, or, in their function. The identification and functional expression of other members of the GluCl family such as those encoded by cosmids C27H5.8 and ZC317.5 in *C. elegans* would therefore be relavant. Orthologues of these genes can in turn be identified and characterised in the parasitic nematodes.

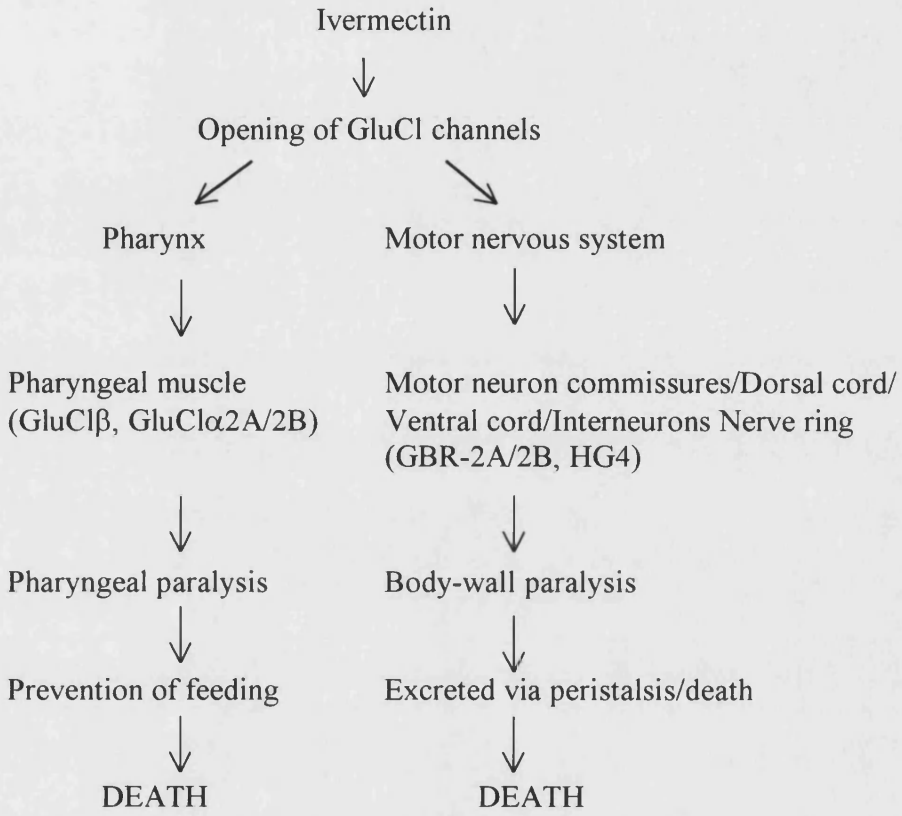


Figure 7.1 A model for the action of ivermectin on the glutamate-gated chloride (GluCl) ion channel receptors. Although GluCl receptors are a target for ivermectin, whether it kills worms by preventing feeding is still not clear.

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APPENDIX I

Degenerate primers used in PCR amplification of the partial receptor subunits HG1-HG5 from *Haemonchus contortus* and Asg2 from *Ascaris suum* (Laughton *et. al.*, 1994).

Primer	*Sequence*	Tm (°C)
DL11	5′ (A/C)T(C/T)TGGGTGCCAGACACCT(A/T)(C/T)TT 3′	66-72
DL19	5′ (A/G)(A/G)(A/G)TTNA(A/G/T)CCA(A/G)AAN(C/G)(A/T)NACCCA 3′	68-86

APPENDIX II

Sequence alignment of members of the glutamate-gated chloride (GluCl) ion channel family of receptor subunits using the GCG program Pileup (Devereux *et. al.*, 1987). The two cysteine residues forming a di-sulphide bond are shown in bold. Approximate regions of the transmembrane domains are underlined.

```
GapWeight: 3.000
GapLengthWeight: 0.100
```

```
  Name: HG4              Len:   746  Check: 9149  Weight:  1.00
  Name: GluClβ           Len:   746  Check: 7172  Weight:  1.00
  Name: GluClα2B         Len:   746  Check: 8361  Weight:  1.00
  Name: GluClα2A         Len:   746  Check: 6163  Weight:  1.00
  Name: GluClα1          Len:   746  Check: 2161  Weight:  1.00
  Name: HcGBR2B          Len:   746  Check: 1188  Weight:  1.00
  Name: CeGBR2B          Len:   746  Check: 2210  Weight:  1.00
  Name: AsGBR2           Len:   746  Check: 4633  Weight:  1.00
  Name: HcGBR2A          Len:   746  Check: 2693  Weight:  1.00
  Name: CeGBR2A          Len:   746  Check: 7452  Weight:  1.00
  Name: OvGluCl          Len:   746  Check: 5971  Weight:  1.00
  Name: HG5              Len:   746  Check: 7249  Weight:  1.00
  Name: ZC317            Len:   746  Check: 1055  Weight:  1.00
  Name: DmGluCl          Len:   746  Check: 6478  Weight:  1.00
  Name: C27H5            Len:   746  Check: 1977  Weight:  1.00
```

```
            1                                                  50
       HG4  .......... .......... .......... .......... ..........
    GluClβ  .......... .......... .......... .......... ..........
  GluClα2B  .......... .......... .......... .......... ..........
  GluClα2A  MIGRLRRGFI LFPQLFLLFV SISSIFLFVL VECKPPKSLN RMRSKGTYNA
   GluClα1  .......... .......... .......... .......... ..........
   HcGBR2B  .......... .......... .......... .......... ..........
   CeGBR2B  .......... .......... .......... .......... ..........
    AsGBR2  .......... .......... .......... .......... ..........
   HcGBR2A  .......... .......... .......... .......... ..........
   CeGBR2A  .......... .......... .......... .......... ..........
   OvGluCl  .......... .......... .......... .......... ..........
       HG5  .......... .......... .......... .......... ..........
     ZC317  .......... .......... .......... .......... ..........
   DmGluCl  .......... .......... .......... .......... ..........
     C27H5  .......... .......... .......... .......... ..........
```

```
          51                                                  100
     HG4  .......... .......... .......... .......... ..........
   GluClβ .......... .......... .......... .......... ..........
GluClα2B  .......... .......... .......... .......... ..........
GluClα2A  AAFNSPSMIN NGLLLAGFEN NDTSRETRES YEEYKDDIDH LLEGGDLTFY
  GluClα1 .......... .......... .......... .......... ..........
  HcGBR2B .......... .......... .......... .......... ..........
  CeGBR2B .......... .......... .......... .......... ..........
   AsGBR2 .......... .......... .......... .......... ..........
  HcGBR2A .......... .......... .......... .......... ..........
  CeGBR2A .......... .......... .......... .......... ..........
  OvGluCl .......... .......... .......... .......... ..........
     HG5  .......... .......... .......... .......... ..........
    ZC317 .......... .......... .......... .......... ..........
  DmGluCl .......... .......... .......... .......... ..........
    C27H5 .......... .......... .......... .......... ..........

          101                                                 150
     HG4  .......... .......... .......... .......... ..........
   GluClβ .......... .......... .......... .......... ..........
GluClα2B  .......... .......... .......... .......... ..........
GluClα2A  DEGADTAAGD VVITSETPVE KHKEVHFKNE PEEEEIGKED DGGGEAEEGE
  GluClα1 .......... .......... .......... .......... ..........
  HcGBR2B .......... .......... .......... .......... ..........
  CeGBR2B .......... .......... .......... .......... ..........
   AsGBR2 .......... .......... .......... .......... ..........
  HcGBR2A .......... .......... .......... .......... ..........
  CeGBR2A .......... .......... .......... .......... ..........
  OvGluCl .......... .......... .......... .......... ..........
     HG5  .......... .......... .......... .......... ..........
    ZC317 .......... .......... .......... .......... ..........
  DmGluCl .......... .......... .......... .......... ..........
    C27H5 .......... .......... .......... .......... ..........

          151                                                 200
     HG4  .......... .......... .......... .......... ..........
   GluClβ .......... .......... .......... .......... ..........
GluClα2B  .......... .......... MSTSFIRRLA FV.....GLL LGV.....HA
GluClα2A  YEEENGSDAE EEEESPEKVE PATSTITTEA QTTTTPEEVT QDVSDNIEDD
  GluClα1 .......... .......... MATWIVGKLI IA.....SLI LGI.....QA
  HcGBR2B .......... .......... .......... .......... ..........
  CeGBR2B .......... .......... .......... .......... ..........
   AsGBR2 .......... .......... .......... .......... ..........
  HcGBR2A .......... .......... .......... .......... ..........
  CeGBR2A .......... .......... .......... .......... ..........
  OvGluCl .......... .......... .......... .......... ..........
     HG5  .......... .......... .......... .......... ..........
    ZC317 .......... .......... .......... .......... ..........
  DmGluCl .......... .......... .......... .......... ..........
    C27H5 .......... ........MF SSVFNFQQFL FGILIRLCLF VDLDDLHNPS
```

```
          201                                                       250
     HG4  .......... .......... .........M SQYMMVAVAA VVAVAGSSQI
   GluClβ .......... .......... ......MTTP SSFSILLLLL LMPVVTNGEY
GluClα2B YHSRPKSEEH SPKHAASSSD LFEDDDQ..S TTLESIARLS APAHVPIEQP
GluClα2A EDARPKSEEH SPKHAASSSD LFEDDDQ..S TTLESIARLS APAHVPIEQP
 GluClα1 QQARTKSQ.. .........D IFEDDNDNGT TTLESLARLT SPIHIPIEQP
  HcGBR2B .......... ........MR NSVPLATRIG PMLALICTVS TIMSAVEAKR
  CeGBR2B .......... .......... ...MWHYRLT TILLIIS... .IIHSIRAKR
   AsGBR2 .......... .......... .......... .......... ..........
  HcGBR2A .......... ........MR NSVPLATRIG PMLALICTVS TIMSAVEAKR
  CeGBR2A .......... .......... ...MWHYRLT TILLIIS... .IIHSIRAKR
  OvGluCl .......... .......... .........M NSFPIVCWNL AFLILVVAKK
     HG5  .......... .......... ........MF ALILPFLLHF TRSEGFGYEK
    ZC317 .......... .......... .......MSL RSLLNILLIV AFWIVGGNCD
   DmGluCl .......... .......... ...MGSGHYF WAILYFASLC SASLANNAKV
     C27H5 TDHLPLVPSR RESARNAKET VYHRLFFVMK AQLYVSVLLA LLVSSTAKKS

          251                                                       300
     HG4  SRRSTGGTQE .......... ..QEILNELL SNYDMRVRPP PSNYSDPM..
   GluClβ SMQS.....E .......... ..QEILNALL KNYDMRVRPP PAN.SSTE..
GluClα2B QTSDTE..IL .......... ..EHLLT... RGYDHRVRPP G.....EDGT
 GluCl2A QTSDTE..IL .......... ..EHLLT... RGYDHRVRPP G.....EDGT
 GluCl1  QTSDSK..IL .......... ..AHLFT... SGYDFRVRPP T.....DN..
  HcGBR2B KLKEQE..II .......... ..QRILN... .NYDWRVRPR GLNASWPDTG
  CeGBR2B KLKEQE..II .......... ..QRILK... .DYDWRVRPR GMNATWPDTG
   AsGBR2 .......... .......... .......... .......... .......DTG
  HcGBR2A KLKEQE..II .......... ..QRILN... .NYDWRVRPR GLNASWPDTG
  CeGBR2A KLKEQE..II .......... ..QRILK... .DYDWRVRPR GMNATWPDTG
  OvGluCl KLKEQE..II .......... ..QRTLK... .DYDWRVRPR GNNLSWPDTG
     HG5  LLDEQK..II .......... ..KHLLESPY SDYDWRVRPR GRLGPADDDD
    ZC317 ASSDTE..II .......... ..KKLLG... KGYDWRVRPP GINLTIPGT.
   DmGluCl NFREKEKKVL .......... ..DQILGA.. GKYDARIRPS GINGT.....
     C27H5 KTKSCKRTAF SRHTTNYQAW REQMTVCDLL QDYDAAVRPS G....RTPYN

          301                                                       350
     HG4  ...GPVTVRV NIMIRMLSKI DVVNMEYSMQ LTFREQWLDS RLAYAHLG..
   GluClβ ...GAVNVRV NIMIRMLSKI DVVNMEYSIQ LTFREQWIDP RLAYENLG..
GluClα2B IHGGPVVVSV NMLLRSISKI DNVNMEYSVQ LTFRESWVDK RLSFGVKGDA
GluClα2A IHGGPVVVSV NMLLRSISKI DNVNMEYSVQ LTFRESWVDK RLSFGVKGDA
 GluClα1 ..GGPVVVSV NMLLRTISKI DVVNMEYSAQ LTLRESWIDK RLSYGVKGDG
  HcGBR2B ...GPVLVTV NIYLRSISKI DDVNMEYSAH FTFREEWVDA RLAYGRFEDE
  CeGBR2B ...GPVLVTV NIYLRSISKI DDVNMEYSAQ FTFREEWTDQ RLAYERYEES
   AsGBR2 ...GPVLVSV NIYLRSISKI DDVNMEYSAQ FTFREEWRDA RLAYERFADE
  HcGBR2A ...GPVLVTV NIYLRSISKI DDVNMEYSAQ FTFREEWVDA RLAYGRFEDE
  CeGBR2A ...GPVLVTV NIYLRSISKI DDVNMEYSAQ FTFREEWTDQ RLAYERYEES
  OvGluCl ...GPVLVSV NIYLRSISKI DDVNMEYSAQ FTFREEWNDA RLGYERLADE
     HG5  YDSEPVFITV NMYLRSISKV DDVNMEYSLH FTFREEWIDE RLYF......
    ZC317 ..HGAVIVYV NMLIRSISKI DDVNMEYSVQ LTFREEWVDG RLAYGFPGDS
   DmGluCl ..DGPAIVRI NLFVRSIMTI SDIKMEYSVQ LTFREQWTDE RLKFDDIQGR
     C27H5 DTRGAVMVTT SLNIRSISAV SEKNMEFVAQ FRFRQEWYDD RLRFIEHQGL
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          351                                                  400
     HG4  .YHNPPKFLT VP.....HIK SNLWIPDTFF PTEKAAHRHL IDTDNMF.LR
   GluClβ  .FYNPPAFLT VP.....HVK KSLWIPDTFF PTEKAAHRHL IDMENMF.LR
GluClα2B  Q....PDFLI LTAG..... QEIWMPDSFF QNENQAYKHM IDKPNVL.IR
GluClα2A  Q....PDFLI LTAG..... QEIWMPDSFF QNENQAYKHM IDKPNVLIIR
 GluClα1  Q....PDFVI LTVG..... HQIWMPDTFF PNEKQAYKHT IDKPNVL.IR
 HcGBR2B  S.TEVPPFVV LATSENADQS QQIWMPDTFF QNEKEARRHL IDKPNVL.IR
 CeGBR2B  GDTEVPPFVV LATSENADQS QQIWMPDTFF QNEKEARRHL IDKPNVL.IR
  AsGBR2  .NTQVPPFVV LATSEQADLT QQIWMPDTFF QNEKEARRHL IDKPNVL.IR
 HcGBR2A  S.TEVPPFVV LATSENADQS QQIWMPDTFF QNEKVARRHL IDKPNVL.IR
 CeGBR2A  GDTEVPPFVV LATSENADQS QQIWMPDTFF QNEKEARRHL IDKPNVL.IR
 OvGluCl  .NTQVPPFVV LAASEQPDLT QQIWMPDTFF QNEKEARRHL IDKPNVL.IR
     HG5  .NSPTLKHIV LSPG..... QRIWVPDTFF QNEKDGKKHD IDTPNIL.IR
    ZC317  ....TPDFLI LTAG..... QQIWMPDSFF QNEKQAHKHD IDKPNVL.IR
 DmGluCl  .....LKYLT LTEA..... NRVWMPDLFF SNEKEGHFHN IIMPNVY.IR
    C27H5  LSSDYRNFEF IHVARD.... QSLWIPDTFF QNEKNGWYHM LNQENRF.LK

          401                                                  450
     HG4  IHPD.GKVLY SSRISITSSC HMQLQLYPLD LQFCDFDLVS YAHTMKDIVY
   GluClβ  IYPD.GKILY SSRISLTSSC PMRLQLYPLD YQSCNFDLVS YAHTMNDIMY
GluClα2B  VHKD.GTILY SVRISLVLSC PMHLQYYPMD VQQCFIDLAS YAYTTKDIEY
GluClα2A  VHKD.GTILY SVRISLVLSC PMHLQYYPMD VQQCFIDLAS YAYTTKDIEY
 GluClα1  IHND.GTVLY SVRISLVLSC PMYLQYYPMD VQQCSIDLAS YAYTTKDIEY
 HcGBR2B  IHKD.GSILY SVRLSLVLSC PMSLEFYPLD RQNCLIDLAS YAYTTQDIKY
 CeGBR2B  IHKN.GQILY SVRLSLVLSC PMSLEFYPLD RQNCLIDLAS YAYTTQDIKY
  AsGBR2  IHQD.GQILY SVRLSLVLSC PMSLEYYPLD RQTCLIDLAS YAYTTDDIKY
 HcGBR2A  IHKD.GSILY SVRLSLVLSC PMSLEFYPLD RQNCLIDLAS YAYTTQDIKY
 CeGBR2A  IHKN.GQILY SVRLSLVLSC PMSLEFYPLD RQNCLIDLAS YAYTTQDIKY
 OvGluCl  IHPD.GQILY SVRLSLVLSC PMSLEYYPLD RQTCLIDLAS YAYTTDDIKY
     HG5  IHNGTGKILY SCRLTLTLSC PMRLADYPLD VQTCVVDFAS YAYTTKDIEY
    ZC317  IHRD.GRILY SVRISMVLSC PMHLQYYPMD VQTCLIDLAS YAYTENDIEY
 DmGluCl  IFPN.GSVLY SIRISLTLAC PMNLKLYPLD RQICSLRMAS YGWTTNDLVF
    C27H5  IRSD.GKLIY DRRLTLHLAC SMHLSRYPMD HQNCEIAFAS YAYTTADIEY

          451                                                  500
     HG4  EWDPLAPVQL KPGVGSDLPN FQLTNITTND DCTSHTNTGS YACLRMQLTL
   GluClβ  EWDPSTPVQL KPGVGSDLPN FILKNYTTNA DCTSHTNTGS YGCLRMQLLF
GluClα2B  VWKEETPVQL KAGLSSSLPS FQLTNTST.T YCTSKTNTGS YSCLRTIIQL
GluClα2A  VWKEETPVQL KAGLSSSLPS FQLTNTST.T YCTSKTNTGS YSCLRTIIQL
 GluClα1  LWKEHSPLQL KVGLSSSLPS FQLTNTST.T YCTSVTNTGI YSCLRTTIQL
 HcGBR2B  EWKEQNPVQQ KDGLRQSLPS FELQDVVT.K YCTSKTNTGE YSCARVKLLL
 CeGBR2B  EWKEKKPIQQ KDGLRQSLPS FELQDVVT.D YCTSLTNTGE YSCARVVLRL
  AsGBR2  EWKLTNPIQQ KEGLRQSLPS FELQDVLT.D YCTSKTNTGE YSCARVKLLL
 HcGBR2A  EWKEQNPVQQ KDGLRQSLPS FELQDVVT.K YCTSKTNTGE YSCLRTQMVL
 CeGBR2A  EWKEKKPIQQ KDGLRQSLPS FELQDVVT.D YCTSLTNTGE YSCLRTRMVL
 OvGluCl  EWKVKNPIQQ KEGLRQSLPS FELQDVLT.E YCTSKTNTGE YSCARVLLLL
     HG5  GWKEEKPIQI KDGLRQSLPS FLLSNVKT.G NCTSVTNTGA YSCLRTIIEL
    ZC317  RWKKTDPVQL KKGLHSSLPS FELNNVSS.. .......... .......FKI
 DmGluCl  LWKEGDPVQV VKNLH..LPR FTLEKFLT.D YCNSKTNTGE YSCLKVDLLF
    C27H5  IW.DVPAIQI HEGANGALPN FEIASFKNAS .CTSKTNTGT YSCLKVEIRL
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          501                                                  550
    HG4   KRQFSYYLVQ LYGPTTMIVI VSWVSFWIDM HSTAGRVALG VTTLLTMTTM
  GluClβ  KRQFSYYLVQ LYAPTTMIVI VSWVSFWIDL HSTAGRVALG VTTLLTMTTM
 GluClα2B RRQFSYYLLQ LYIPSCMLVI VSWVSFWIDR TAVPARVTLG VTTLLTMTTQ
 GluClα2A RRQFSYYLLQ LYIPSCMLVI VSWVSFWIDR TAVPARVTLG VTTLLTMTTQ
  GluClα1 KREFSFYLLQ LYIPSCMLVI VSWVSFWFDR TAIPARVTLG VTTLLTMTAQ
  HcGBR2B RREYSYYLIQ LYIPCIMLLV VSWVSFWLDK DAVPARVSLG VTTLLTMTTQ
  CeGBR2B RREYSYYLIQ LYIPCIMLVV VSWVSFWLDK DAVPARVSLG VTTLLTMTTQ
   AsGBR2 RREYSYYLIQ LYIPCIMLVV VSWVSFWLDK DAVPARVSLG VTTLLTMTTQ
  HcGBR2A RREFSYYLLQ LYIPSFMLVI VSWVSFWLDK DSVPARVTLG VTTLLTMTTQ
  CeGBR2A RREFSYYLLQ LYIPSFMLVI VSWVSFWLDK DSVPARVTLG VTTLLTMTTQ
  OvGluCl RREYR..... .......... .......... .......... ..........
    HG5   KREFSYYLLQ LYIPSFMLVA VSWVSFWLDK XSVPARVTLG VTTLLTMTTQ
   ZC317  RKRLSY.... .......... .......... .......... ..........
  DmGluCl RREFSYYLIQ IYIPCCMLVI VSWVSFWLDQ GAVPARVSLG VTTLLTMATQ
   C27H5  NRVFSFFLLQ LYIPSSMLVG VAWVSYWIDW KSTAARVPLA IVTLLTMITT
          ———————— TM I ———————     ———————————— TM II

          551                                                  600
    HG4   QAAINAKLPP VSYVKVVDVW LGACQTFVFG ALLE.YAFVS YQDSQRQTEQ
  GluClβ  QSAINAKLPP VSYVKVVDVW LGACQTFVFG ALLE.YAFVS YQDSVRQNDR
 GluClα2B SSGINAKLPP VAYIKAIDVW IGACMTFIFC ALLE.FAWVT YIAN.KQDAN
 GluClα2A SSGINAKLPP VAYIKAIDVW IGACMTFIFC ALLEFFAWVT YIANKKQDAN
  GluClα1 SAGINSQLPP VSYIKAIDVW IGACMTFIFC ALLE.FALVN HIAN.KQGVE
  HcGBR2B ASGINSKLPP VSYIKAVDVW IGVCLAFIFG ALLE.YAVVN YYGRKEFLRK
  CeGBR2B ASGINTKLPP VSYIKAVDVW IGVCLAFIFG ALLE.YAVVN YYGRKEFLRK
   AsGBR2 ASGINSKLPP VSYIKAVDVW IGVCLAFIFG ALLE.YALVN YHGRQEFLKK
  HcGBR2A SSGINANVPP VSYTKAIDVW IGVCLAFIFG ALLE.FAWVN YAARKDM...
  CeGBR2A SSGINANVPP VSYTKAIDVW IGVCLAFIFG ALLE.FALVN YAARKDMTQ.
  OvGluCl .......... .......... .......... .......... ..........
    HG5   ASGVNANLPP VSYTKAIDIW IGVCLAFIFG ALLE.FALVN WAARQDLVAH
   ZC317  ASGINAKLPP VSYTKAIDVW IGACLTFIFG ALLE.FAWVT YISSRSFYKR
  DmGluCl TSGINASLPP VSYTKAIDVW TGVCLTFVFG ALLE.FALVN YASRSGSNKA
   C27H5  SHAINSNLPP VSYAKSIDIW VGACVVFIFF SLIE.YAVVN YVGILDEHRQ
          ———— ———————— TM III ————————

          601                                                  650
    HG4   .AKSRAARKA QKRRAKMELV ER..... .......... ..........
  GluClβ  .SREKAARKA QRRREKLEMV DA..... .......... ..........
 GluClα2B .KRARTEREK AELPFLQNSH NDVWVPRE.. .......... .........VA
 GluClα2A .KRARTEREK AELPFLQNSH NDVWVPRE.. .......... .........VA
  GluClα1 .RKARTEREK AEIPLLQNLH ND..VPTK.. .......... .........VF
  HcGBR2B .EKKKKTRLD DCVCPSE... ....RPAL.. .......... .........RL
  CeGBR2B .EKKKKTRID DCVCPSD... ....RPPL.. .......... .........RL
   AsGBR2 .EKKKKTGLQ ECLCPNDQPL TQGAHPIT.. .......... .........RL
  HcGBR2A .......... .......... SCGQRMMK.. .......... .........QL
  CeGBR2A .......... .........V SQRIRQMK.. .......... .........QL
  OvGluCl .......... .......... .......... .......... ..........
    HG5   .SRARYRQSP LFFRNPDSRQ GNSHHFYA.. .......... .........PI
   ZC317  .NKNCSSRNS LLIETKQALI IPNTVVAQ.. .......... .........FP
  DmGluCl .NMHKENMKK KRRDLEQASL DAASDLLDTD SNATFAMKPL VRHPGDPLAL
   C27H5  MKKAACNRSR LSNVIENDNF GESLQSLTFS PQEKKRLIRR RPKKNMEMQE
```

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          651                                                    700
     HG4  EQYQPPCTCH LYQDYEPSFR DRLRRYFT.. .......... ..........
   GluClβ EVYQPPCTCH TFEARE.TFR DKVRRYFT.. .......... ..........
GluClα2B  EQEREVMTVR MNRRQTNSVW KWIKTKTE.. .......... ..........
GluClα2A  EQEREVMTVR MNRRQTNSVW KWIKTKTE.. .......... ..........
 GluClα1  NQEEKVRTVP LNRRQMNSFL NLLETKTE.. .......... ..........
 HcGBR2B  DLSNYRRRGW TP.LNRL..L DMLGR..... .......... ..........
 CeGBR2B  DLSAYRSVKR LPIIKRI..S EILST..... .......... ..........
  AsGBR2  DMSVYRKRKL LN.MPGL..R AWFSS..... .......... ..........
 HcGBR2A  PQDGYRPLAG SQPRTSFCCR IFVRR..... .......... ..........
 CeGBR2A  PTEGYRPLSA SQGRSSFCCR IFVRR..... .......... ..........
 OvGluCl  .......... .......... .......... .......... ..........
     HG5  QQE....VTL EDLPFSWWDK IWKIR..... .......... ..........
   ZC317  EHPEESYFLL SSYVSTLSTG PSIHNITH.. .......... ..........
 DmGluCl  EKRLQCEVHM QAPKRPNCCK TWLSKFPT.. .......... ..........
   C27H5  GDFEAIEMVD RGPPRSAGLM EEGWTFHDTT DLVYIGQRKR VELVRWCSVL
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          701                                                746
     HG4  KPDYLPAKID YYARFCVPLG FLAFNAIYWT SCLVMVSRLV ......
   GluClβ KPDYLPAKID FYARFVVPLA FLAFNVIYWV SCLIMSANAS TPESLV
GluClα2B  WN.DKSKRAD LISRVMFPVL FLTFNISYWT HYGQYGVAIS T.....
GluClα2A  WN.DKSKRAD LISRVMFPVL FLTFNISYWT HYGQYGVAIS T.....
 GluClα1  WN.DISKRVD LISRALFPVL FFVFNILYWS RFGQQNVLF. ......
 HcGBR2B  NA.DLSRRVD LMSRITFPSL FTAFLVFYYS VYVKQSNLD. ......
 CeGBR2B  NI.DISRRVD LMSRLTFPLT FFSFLIFYYV AYVKQSRD.. ......
  AsGBR2  TS.EVSKRVD LISRFTFPSF FTCFLVFYYV TYVK...... ......
 HcGBR2A  YK.ERSKRID VVSRLVFPIG YACFNVLYWA VYLM...... ......
 CeGBR2A  YK.ERSKRID VVSRLVFPIG YACFNVLYWA VYLM...... ......
 OvGluCl  .......... .......... .......... .......... ......
     HG5  YK.ERSRRID LISRVMFPLC FIIFNIMYWW RYLIPYMAVQ AQLE..
   ZC317  RK.P.NEIVQ LISKF..... .......... .......... ......
 DmGluCl  RQCSRSKRID VISRITFPLV FALFNLVYWS TYLFREEEDE ......
   C27H5  SSRGRAERID IIARIIFPLA FILFNFAYWS IYLEEEDPDE S.....
```

TM IV

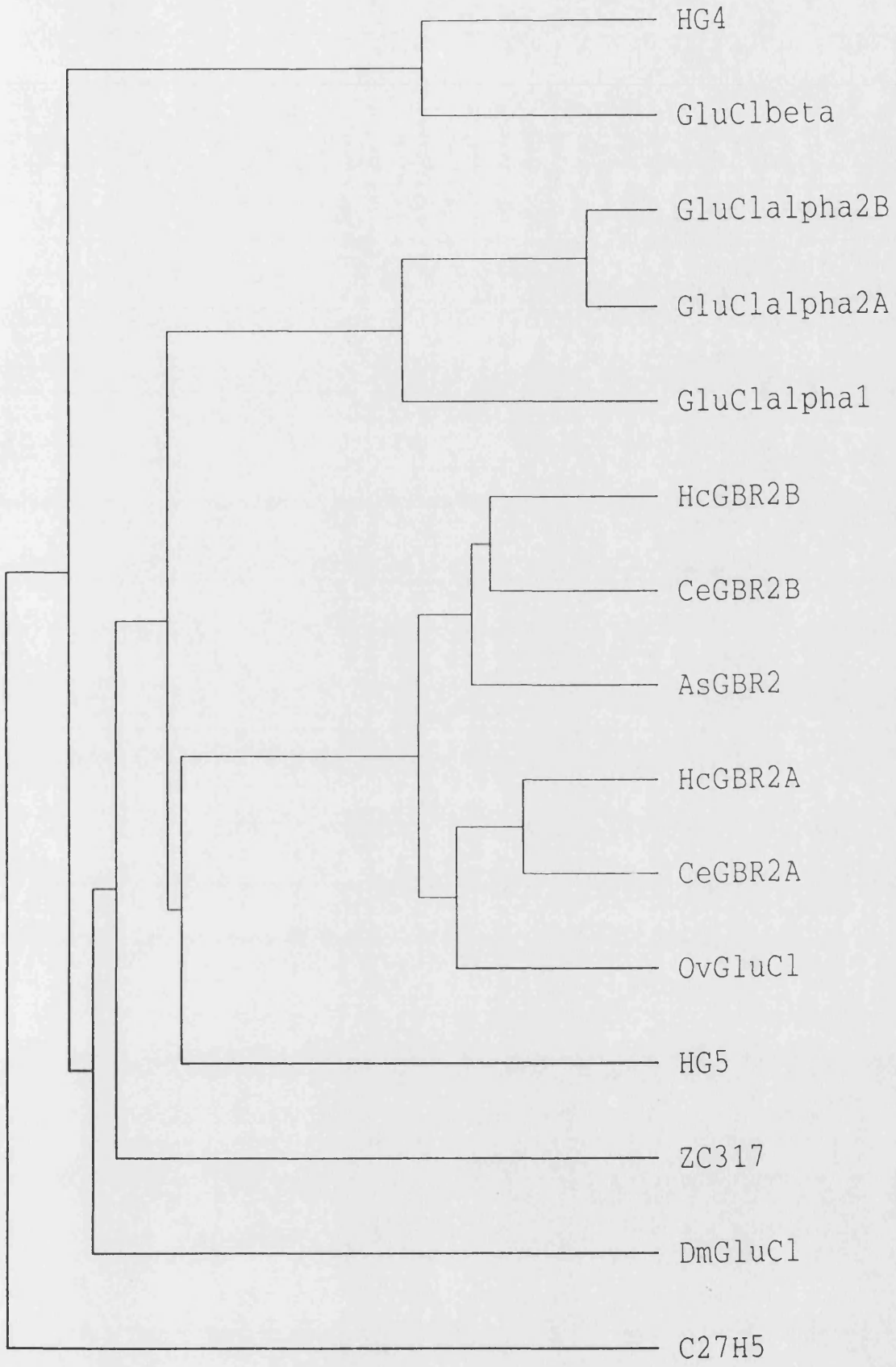
APPENDIX III

1 HG4
2 GluClβ
3 GluClα2B
4 GluClα2A
5 GluClα1
6 Hc-GBR-2B
7 Ce-GBR-2B
8 As-GBR-2
9 Hc-GBR-2A
10 Ce-GBR-2A
11 OvGluCl
12 HG5
13 ZC317.5
14 DmGluCl
15 C27H5.8

	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	77	47	46	46	45	44	47	48	47	47	41	42	41	36
2		46	46	43	45	44	47	48	47	49	41	44	43	37
3			96	75	52	53	57	57	57	58	53	59	45	34
4				74	53	54	57	57	57	58	53	59	45	33
5					52	53	57	55	56	56	52	53	43	35
6						84	84	83	74	81	54	52	48	38
7							82	74	82	81	55	51	49	39
8								74	73	94	58	55	52	40
9									89	79	61	53	51	41
10										79	62	53	52	41
11											57	60	46	40
12												46	47	41
13													41	31
14														35

Table of percent amino acid identities of members of the glutamate-gated chloride (GluCl) ion channel family of receptor subunits. Values of the matrix were obtained using the DISTANCES program in GCG and subtracting from 100 (Devereux *et. al.*, 1984).

APPENDIX IV



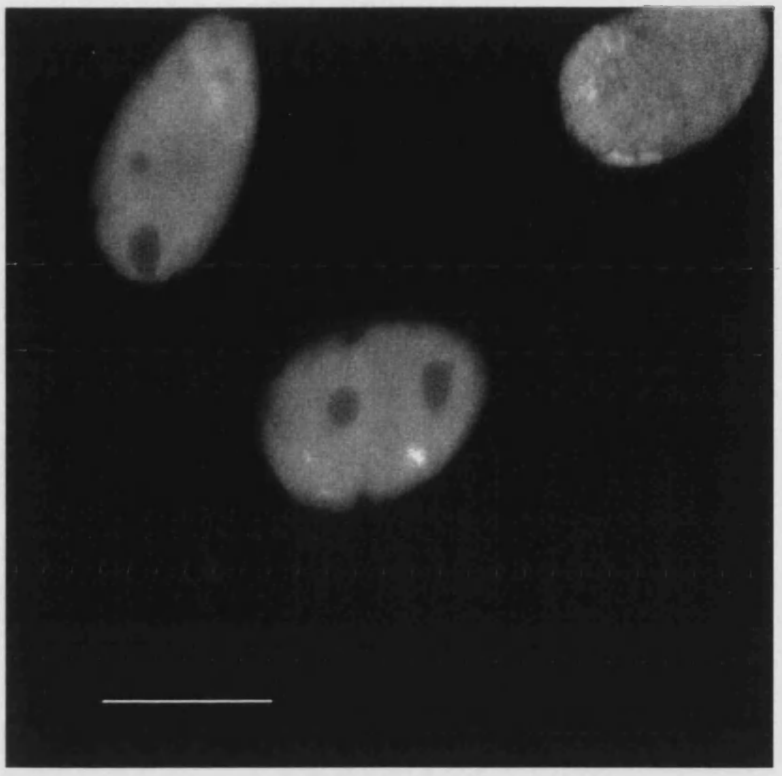
The table of amino acid identities of sequences of the glutamate-gated chloride (GluCl) ion channel family in Appendix III is presented as a tree. This was generated using the 'PILEUP' program in GCG (Devereux *et. al.*, 1984).

APPENDIX V

Images taken with a Zeiss LSM 510 confocal fitted to an inverted Axiovert 100M confocal microscope, using a helium neon laser and rhodamine filter set.

(A) Confocal image at high resolution of *Haemonchus contortus* eggs. The adult female is a prolific egg-layer. It lays about 10,000-15000 eggs per day.

(B) Confocal image of a somatic muscle cell from *Ascaris suum*. The cell is composed of a contractile spindle (s), an enlarged balloon-shaped bag (b) containing the nucleus and glycogen granules and; the arm (a) which is a thin process at the base of the bag and leads to the syncytium (sy). The *Ascaris* muscle cells are peculiar in that branches of the muscle pass to the nervous system rather than processes of the nervous system passing to the muscle cells.



A

B

APPENDIX VI

Table showing the plasmid constructs during this study.

Plasmid	Genotype	Media
pASJ1.1, pASJ1.8	pBluescript */Xba1/Xho1* with 450bp partial receptor subunit As-GBR-2 from *Ascaris suum*	LB+ampicillin (100 µg/ml)
pASJ2.1, pASJ2.2	pBluescript / *SmaI* with 3′ RACE-PCR product of the 450bp partial receptor subunit Asg2 from *Ascaris suum*	LB+ampicillin (100 µg/ml)
pH2BSJ2.1, pH2BSJ2.7	Full length clone of Hc-GBR-2B from *Haemonchus contortus*	LB+ampicillin (100 µg/ml)